A rapid HPLC–ESI-MS/MS for qualitative and quantitative analysis of saponins in ‘XUESETONG’ injection

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Abstract
‘XUESETONG’ injection, one of the most widely used proprietary medicines in traditional Chinese medicine, consists of total saponins made from Panax notoginseng, which is a highly valued and important Chinese medicinal herb. It is used to treat cardiovascular diseases. In order to control the quality of XUESETONG injection, a rapid HPLC–ESI-MS/MS method was developed for qualitative and quantitative determination of the saponins. The analyses were performed on SB-C18 column using gradient elution in 25 min. Full scan and time programmed selected reaction monitoring (SRM) were used for qualitative and quantitative analysis of saponins, respectively. Twenty-seven saponins were identified and nine of them including notoginsenoside R1, ginsenoside Rb1, Rb2, Rh1, Rc, Rd, Re, Rf and Rg1 were quantified. Ten XUESETONG injections were analyzed and compared. The results showed that there is a great variation among different samples. In conclusion, the developed method is rapid, accurate and sensitive for qualitative and quantitative analysis of saponins in XUESETONG injection. Moreover, it also can be used for the quality control of Panax notoginseng raw material and its preparations.

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Keywords: XUESETONG injection; Panax notoginseng; Total saponins; HPLC–ESI-MS/MS; Quality control; Notoginsenoside; Ginsenoside

1. Introduction
‘XUESETONG’ injection consists of total saponins made from Panax notoginseng (commonly known as Tiaoli or Sanqi), which is a highly valued and important Chinese medicinal herb produced mainly in Yunnan Province of China, is one of the most widely used proprietary medicines in traditional Chinese medicine. It is used to treat cardiovascular diseases. The dammarane-type saponins, which include ginsenosides and notoginsenosides, contribute to pharmacological activity [1–4]. In general, the curative effect of traditional Chinese medicine is an integrative result of a number of bioactive compounds. Therefore, analysis of saponins is helpful to control the quality of XUESETONG injection. Actually, several methods, including HPTLC [5–7], HPLC–UV [8–10], HPLC–ELSD [11], have been used for analysis of saponins in Panax ginseng. However, these methods suffer from low resolution and reproducibility [5–7] or low sensitivity and long analytical time [8–11]. Up to date, qualitative identification and quantitative determination of saponins in Panax notoginseng were not performed by HPLC–MS, though the technique has been applied to identify Panax notoginseng from Panax ginseng and Panax quinquefolius [12] and the saponins in P. ginseng [13–16]. On the other hand, the analytical time of most previous studies was more than 60 min [12,15,16]. In our current study, a rapid HPLC–ESI-MS/MS method was first attempted to analyze saponins in XUESETONG injection. Using this method, 27 saponins were identified and nine saponins were quantitatively determined in 25 min.

2. Experimental
2.1. Chemicals, standards and samples
Acetone for liquid chromatography was purchased from Merck (Darmstadt, Germany). Ammonium acetate was purchased from Riedel-de Haën (Seelze, Germany). Chemicals not mentioned here were from standard sources. Water was prepared using a Millipore Mili Q-Plus system (Millipore, Bedford, MA). The standards of ginsenoside Rb1, Rb2, Rc, Rd, Rf and Rg1 were purchased from ChromaDex Company (Santa, 0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
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Ana, USA). Notoginsenoside R1 was kindly supplied by Kun- 
ming Institute of Botany, Scientific Academy of China (Kun-
ming, China). The standard saponins were first dissolved in 
initial mobile phase (8 mM ammonium acetate aqueous solu-
tion/acetonitrile; 8/2; v/v) at ~0.5 mg/ml as a stock solution. 
1 ml of the stock solution was transferred to a 2 ml volumetric 
flask, which was brought up to its volume with the mobile phase. 
A series dilution was performed so as to obtain the desired con-
centration. Filtered through a 0.45 μm membrane (Econo
ther RC, Agilent Technologies), a certain volume of standard solu-
tion was injected for LC–MS analysis.

Ten samples of XUESETONG injection (25–50 mg/ml) were 
collected in the market. They were manufactured by four phar-
maceutical companies in China, named A, B, C and D, respec-
tively. The samples were deposited at the Institute of Chinese 
Medical Sciences, University of Macau, Macau SAR, China. 
A certain volume of the injection, according to its nominal 
content of total saponins, was transferred to a 50 ml volumet-
ric flask which was brought up to its volume with the mobile 
phase so as to obtain the concentration of total saponins at about 
0.5 mg/ml. Filtered through a 0.45 μm membrane, 10 μl of the 
solutions were injected for LC–MS analysis. Spiked injection 
was produced by mixing test sample solution and the reference 
compounds at the ratio of 1:1.

2.2. HPLC–UV–MS analysis

Analysis were performed on an Agilent 1100 Series LC/MSD 
Trap system (Agilent Technologies, Palo Alto, CA), equipped 
with a vacuum degasser, a quaternary pump, an autosampler, 
a column compartment, a DAD detector and an ion-trap mass 
spectrometer with electrospray ionization interface, connected 
to an Agilent LC/MSD Trap Software. A ZORBAX SB-C18 col-
umn (2.1 × 150 mm i.d., 5 μm) and a ZORBAX ODS C18 guard 
column (4.6 × 12.5 mm i.d., 5 μm) were used. Solvents that 
constituted the mobile phase were (A) 8 mM aqueous ammo-
nium-acetate and (B) acetonitrile. The elution conditions applied 
were: 0–3 min, linear gradient 20–25% B; 3–20 min, linear gra-

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Fig. 1. Structure of main saponins in P. notoginseng.

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Fig. 1. (Continued).

dient 25–50% B; 20–30 min, linear gradient 50–80% B; and
finally, reconditioning steps of the column was 20% B isocratic
for 15 min. The flow-rate was 0.4 ml/min and the system oper-
ated at 25 °C. Peaks were detected at 203 nm of UV detection
and negative ion mode of MS and MS/MS detection. ESI-MS
conditions were as follows: drying gas N2 , 7 l/min, tempera-
ture 325 °C, pressure of Nebulizer 25 psi, source voltage 3.5 kV.
ESI-MS/MS conditions: isolation width 4, fragment amplifi-
cation 1.5. Scan range of both ESI-MS and ESI-MS/MS was
200–1400 u.

3. Results and discussion

Nine reference compounds ginsenoside Rb1, Rb2, Rb3, Rc,
Rd, Re, Rf, Rg1 and notoginsenoside R1 (Fig. 1) were ana-
lyzed in order to optimize the MS conditions and calibrate
the quantitative determination. The trials showed that the negative
ion mode was more sensitive than the positive ion mode. The
HPLC–ESI-MS chromatogram exhibited good agreement with
the HPLC–UV chromatogram (Fig. 2). A good chromatographic
separation of saponins in XUESETONG injection was achieved
on reversed-phase using a linear gradient of 8 mM aqueous
ammonium acetate and acetonitrile.

3.1. Qualitative analysis

Fig. 3 A–K showed the 27 peaks detected by using selective
ion monitoring (SIM) in XUESETONG injections. They were
tentatively identified by careful studies of the MS and MS/MS
spectra and by comparison with literature data [15–21]. The
identification of peaks as ginsenosides Rb1, Rb2, Rb3, Rc, Rd,
Re, Rf, Rg1, Rg3 and notoginsenoside R1, respectively; was
also confirmed by spiked injection of the reference compounds.

Example of peaks of \( m/z \) 946 was ginsenoside Re, ginsenoside
Rd, gypenoside XII and ginsenoside Rd isomer (Fig. 3G). The
retention times were 8.1 min (P14), 13.4 min (P15), 16.1 min
(P16) and 16.8 min (P17), respectively. The MS/MS spectra of

Fig. 2. HPLC chromatograms of XUESETONG injection detected by (A) UV and (B) MS detectors.
ginsenosides exhibited a fragmentation pattern corresponding to the loss of the glycosidic units (Table 1, Fig. 4). In addition, an [aglycone-H] ion at \( m/z \) 475 corresponding to the (20S)-protopanaxatriol aglycon moiety was visible for peak P14. Peaks P15, P16 and P17 showed an [aglycone-H] ion at \( m/z \) 459 corresponding to the (20S)-protopanaxadiol aglycon moiety. Peaks P14 and P16 were identified as ginsenosides Re and Rd, confirmed by spiking with standards. Apropos of the peak appear at 13.4 min, the MS data had \( m/z \) 783 [M–H-Glc]⁻, \( m/z \) 621 [M–H-2Glc]⁻ and \( m/z \) 459 [M–H-3Glc]⁻. The fragment of \( m/z \) 459 was derived from sapogenin produced by the saponin release all linked glucose, which was in accordance with the fragment-
tation pathway of gypenoside XII and ginsenoside Rd isomer. According to the references [12,15], the peak at 16.8 min was identified as the isomer of ginsenoside Rd, which product ion were similar to those of ginsenoside Rd. Therefore, Peak P15 was identified as gypenoside XII.

Using mentioned method above, 27 compounds, including four unknown saponins, as well as ginsenoside F1, Fc (or Ra1/Ra2, Ra3, Rh1, Rb2, Rb3, Rc, Rd, Rd isomer, Re, Rf, Rg1, Rg2, Rg3, Rh1, notoginsenoside F2, Fa, Fe, R1, R2, R4, gypenoside IX and gypenoside XII, were identified (Table 1).
3.2. Quantitative analysis

The number of ions chosen has an effect on the sensitivity of quantitative analysis. The advantage of SIM is achieved through spending more time monitoring the ions of interest—the more ions being monitored, then the less time will be spent on each of them and the lower the increase in sensitivity of SIM over full scanning. When SIM is being carried out in conjunction with chromatography, a further consideration is that an adequate number of cycles of measurement must be made to define the shape and intensity of the chromatographic response exactly, or otherwise inaccurate and imprecise measurements will be made (Fig. 5). In order to increase sensitivity and specificity of quantification, time programmed selective reaction monitoring (SRM) of ginsenoside Rb1 (1108 → 946), ginsenoside Rb2, Rb3, Rc, Rd, Re, Rf, and Rg1, a series of concentration for standards solution were injected and analyzed by LC–ESI-MS/MS under time programmed SRM. This was done by injecting 10 μl of different concentrations on column using the chromatographic conditions described above. All measurements were done in triplicate and data were processed by LC/MSD Trap Software (Version 4.2). The linearity ranges for the investigated compounds were reported in Table 2.

3.2.1. Validation of the method

In order to determine the linearity of notoginsenoside R1, ginsenoside Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, and Rg1, a series of concentration for standards solution were injected and analyzed by LC–ESI-MS/MS under time programmed SRM. This was done by injecting 10 μl of different concentrations on column using the chromatographic conditions described above. All measurements were done in triplicate and data were processed by LC/MSD Trap Software (Version 4.2). The linearity ranges for the investigated compounds were reported in Table 2.
The results showed that the LOD (LOQ) values for nine analytes was between 0.50–4.81% and 0.52–5.58%, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were defined as the signal-to-noise ratio of 3 and 10, respectively. The LOD (LOQ) values were shown in Table 2. The results showed that there was good sensitivity for tested compounds in our calibrated HPLC–ESI-MS and HPLC–ESI-MS/MS system.

In order to determine the accuracy, a known amount of nine investigated compounds was added into the XUESETONG injection. The samples were subjected to LC–ESI-MS/MS. The samples were subjected to LC–ESI-MS/MS ions of saponins in XUESETONG injection (% with relative abundance (% in parentheses)

<table>
<thead>
<tr>
<th>No.</th>
<th>Peak Identification</th>
<th>RT (min)</th>
<th>MS [M–H]−</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P13 Notoginsenoside R1</td>
<td>7.1</td>
<td>932</td>
</tr>
<tr>
<td>2</td>
<td>P8 Ginsenoside Rg1</td>
<td>7.9</td>
<td>800</td>
</tr>
<tr>
<td>3</td>
<td>P14 Ginsenoside Re</td>
<td>8.1</td>
<td>946</td>
</tr>
<tr>
<td>4</td>
<td>P10 Unknown</td>
<td>10.4</td>
<td>916</td>
</tr>
<tr>
<td>5</td>
<td>P25 Ginsenoside Ra/Notoginsenoside F3/unknown</td>
<td>12.3</td>
<td>1240</td>
</tr>
<tr>
<td>6</td>
<td>P18 Notoginsenoside R4/unknown</td>
<td>12.8</td>
<td>1078</td>
</tr>
<tr>
<td>7</td>
<td>P9 Ginsenoside Rf</td>
<td>12.9</td>
<td>800</td>
</tr>
<tr>
<td>8</td>
<td>P26 Ginsenoside Rk/Notoginsenoside F4/unknown</td>
<td>13.6</td>
<td>1240</td>
</tr>
<tr>
<td>9</td>
<td>P11 Notoginsenoside F9/ginsenoside XII</td>
<td>13.1</td>
<td>916</td>
</tr>
<tr>
<td>10</td>
<td>P15 Gypenoside XII</td>
<td>13.4</td>
<td>946</td>
</tr>
<tr>
<td>11</td>
<td>P25 Ginsenoside Rb5</td>
<td>13.5</td>
<td>1108</td>
</tr>
<tr>
<td>12</td>
<td>P3 Notoginsenoside R2/unknown</td>
<td>13.6</td>
<td>770</td>
</tr>
<tr>
<td>13</td>
<td>P27 Ginsenoside Rk/Notoginsenoside F4/unknown</td>
<td>13.7</td>
<td>1240</td>
</tr>
<tr>
<td>14</td>
<td>P19 Ginsenoside Re</td>
<td>14.1</td>
<td>1078</td>
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<td>15</td>
<td>P24 Ginsenoside Fc/Ra1/Ra2</td>
<td>14.2</td>
<td>1210</td>
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<tr>
<td>16</td>
<td>P4 Notoginsenoside R2/unknown</td>
<td>14.3</td>
<td>770</td>
</tr>
<tr>
<td>17</td>
<td>P5 Ginsenoside Rg2</td>
<td>14.7</td>
<td>784</td>
</tr>
<tr>
<td>18</td>
<td>P1 Ginsenoside Fc/Ra1/Ra2</td>
<td>14.8</td>
<td>638</td>
</tr>
<tr>
<td>19</td>
<td>P20 Ginsenoside Rb2</td>
<td>14.9</td>
<td>1078</td>
</tr>
<tr>
<td>20</td>
<td>P21 Ginsenoside Rb3</td>
<td>15.0</td>
<td>1078</td>
</tr>
<tr>
<td>21</td>
<td>P2 Ginsenoside Fc/Ra1/Ra2</td>
<td>15.2</td>
<td>638</td>
</tr>
<tr>
<td>22</td>
<td>P22 Notoginsenoside R2/unknown</td>
<td>15.3</td>
<td>1078</td>
</tr>
<tr>
<td>23</td>
<td>P16 Ginsenoside Rf</td>
<td>16.1</td>
<td>946</td>
</tr>
<tr>
<td>24</td>
<td>P17 Ginsenoside Rf (iso)</td>
<td>16.8</td>
<td>946</td>
</tr>
<tr>
<td>25</td>
<td>P22 Notoginsenoside F3/unknown</td>
<td>18.3</td>
<td>916</td>
</tr>
<tr>
<td>26</td>
<td>P6 Notoginsenoside F2</td>
<td>22.7</td>
<td>784</td>
</tr>
<tr>
<td>27</td>
<td>P7 Ginsenoside Rg3</td>
<td>23.2</td>
<td>784</td>
</tr>
</tbody>
</table>

Table 1
HPLC–ESI-MS and HPLC–ESI-MS/MS ions of saponins in XUESETONG injection (% with relative abundance (% in parentheses)

In order to determine the accuracy, a known amount of nine investigated compounds was added into the XUESETONG injection. The samples were subjected to LC–ESI-MS/MS. The recovery of the tested compounds was within the range of 95.5–100.0%, with R.S.D. for nine analytes was between 2.2 and 5.0%, where n = 3 (Table 2).

3.2.2. Application to quantitative determination of XUESETONG injection

Ten different batches of XUESETONG injection from four pharmaceutical manufacturers were tested. Time programmed SRM chromatograms of nine standard saponins and XUESETONG injection was shown in Fig. 6. Based on the chromatogram, we see that ginsenoside Rg1 and Re, as well as ginsenoside Rb2 and Rb3 cannot be separated, respectively. However, the advantage of the mass spectrometer is that mass ions was relatively stable. The R.S.D. of short and long term repeatability for tested compounds were 0.50–4.81% and 0.52–5.58%, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were defined as the signal-to-noise ratio of 3 and 10, respectively. The LOD (LOQ) values were shown in Table 2. The results showed that there was good sensitivity for tested compounds in our calibrated HPLC–ESI-MS and HPLC–ESI-MS/MS system.
can be used as a discriminating feature and this may allow quantitative measurements to be made on unresolved components. Therefore, individual quantitative determination of ginsenoside Rg1 and Re was available. But total content of ginsenoside Rb2 and Rb3 was determined only because their precursors and ion products were same, and ginsenoside Rb2 was used as calibrating standard. By using the calibration curve of each investigated compound, the amount of analytes in XUESETONG injection was determined within the linear range. Then the calculation was as follows:

\[
\text{Percentage of analyte in XUESETONG injection} = 100 \times \frac{\text{Amount of analyte}}{\text{Nominal amount of total saponins in the injection}}
\]

\[
\text{Percentage of analyte in nine investigated saponins} = 100 \times \frac{\text{Amount of analyte}}{\text{Total amount of nine investigated saponins in the injection}}
\]

Table 2: Linear regression data and validation of developed method for investigated compounds in XUESETONG injection

<table>
<thead>
<tr>
<th>Standard</th>
<th>Target ion (SRM)</th>
<th>Regression equation ( (n=7) ) ( R^2 )</th>
<th>Linear range (( \mu \text{g}/\text{ml} ))</th>
<th>Recovery (%)</th>
<th>LOD (pp/( \mu \text{g} ))</th>
<th>LOQ (pp/( \mu \text{g} ))</th>
<th>Mean R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginsenoside Rb1</td>
<td>1108 → 946</td>
<td>( y = 1312.08x + 660804.72 ) 0.9950</td>
<td>0.0200–1.0900</td>
<td>98.3</td>
<td>2.9</td>
<td>65</td>
<td>809</td>
</tr>
<tr>
<td>Rb2</td>
<td>1078 → 946</td>
<td>( y = 2787.43x + 144301.00 ) 0.9981</td>
<td>0.0026–1.1300</td>
<td>97.5</td>
<td>3.4</td>
<td>104</td>
<td>332</td>
</tr>
<tr>
<td>Re</td>
<td>1078 → 946</td>
<td>( y = 5929.04x + 94044.00 ) 0.9986</td>
<td>0.0002–0.1000</td>
<td>99.6</td>
<td>4.8</td>
<td>100</td>
<td>350</td>
</tr>
<tr>
<td>Rb3</td>
<td>946 → 784</td>
<td>( y = 3004.03x + 744036.43 ) 0.9970</td>
<td>0.0100–0.5000</td>
<td>96.5</td>
<td>4.7</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Rg1</td>
<td>946 → 800</td>
<td>( y = 708.82x + 790501.81 ) 0.9973</td>
<td>0.0488–0.2928</td>
<td>95.5</td>
<td>2.2</td>
<td>122</td>
<td>1220</td>
</tr>
<tr>
<td>Re</td>
<td>800 → 637</td>
<td>( y = 3479.70x + 26254.14 ) 0.9979</td>
<td>0.0128–0.064</td>
<td>100.0</td>
<td>4.2</td>
<td>53</td>
<td>107</td>
</tr>
<tr>
<td>Rg1</td>
<td>800 → 637</td>
<td>( y = 220.22x + 1591821.43 ) 0.9988</td>
<td>0.0350–1.0880</td>
<td>98.8</td>
<td>3.7</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Notoginsenoside R1</td>
<td>932 → 800</td>
<td>( y = 882.85x + 173365.58 ) 0.9948</td>
<td>0.0104–0.3120</td>
<td>98.3</td>
<td>5.0</td>
<td>104</td>
<td>208</td>
</tr>
</tbody>
</table>

\( R^2 \), squares of correlation coefficients for the standard curves; percentage of relative standard deviation (R.S.D.) for three replicates; LOD, limit of detection; LOQ, limit of quantification.
Table 3 shows the summary results. There is a great variation for the contents of investigated saponins in different XUESETONG injection, even though the samples came from the same pharmaceutical factory. Hierarchical cluster analysis was performed based on nine peaks characteristics of investigated saponins in the injection. A method named as average linkage between groups was applied, and Squared Euclidean distance was selected as measurement. In this way, the groups are represented by their mean values for each variable. Fig. 7 shows the results on the tested 10 samples of XUESETONG injection, which are divided into two main clusters. It suggests that there is difference in the quality of XUESETONG injection. Actually, the saponins for XUESETONG injection have two sources, root or rhizomes of *P. notoginseng*. It may be why the quality of XUESETONG injection in different manufacturers or different batches is obviously different.
Table 3: The percentage of individual saponin in nominal amount of total saponins and in total of nine investigated saponins (in parenthesis) from different XUESETONG injection

<table>
<thead>
<tr>
<th>Samples</th>
<th>Notoginsenoside</th>
<th>Ginsenoside</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1</td>
<td>Rg1</td>
</tr>
<tr>
<td>S1</td>
<td>10.50 (10.39)</td>
<td>37.92 (37.51)</td>
</tr>
<tr>
<td>S2</td>
<td>10.43 (10.37)</td>
<td>39.00 (38.78)</td>
</tr>
<tr>
<td>S3</td>
<td>9.89 (9.67)</td>
<td>33.63 (32.88)</td>
</tr>
<tr>
<td>S4</td>
<td>8.12 (8.66)</td>
<td>23.04 (24.57)</td>
</tr>
<tr>
<td>S5</td>
<td>10.38 (10.65)</td>
<td>21.30 (21.26)</td>
</tr>
<tr>
<td>S6</td>
<td>9.12 (9.60)</td>
<td>26.13 (27.51)</td>
</tr>
<tr>
<td>S7</td>
<td>6.63 (7.13)</td>
<td>29.65 (31.33)</td>
</tr>
<tr>
<td>S8</td>
<td>10.25 (10.14)</td>
<td>22.61 (22.36)</td>
</tr>
<tr>
<td>S9</td>
<td>7.62 (7.79)</td>
<td>28.03 (28.67)</td>
</tr>
<tr>
<td>S10</td>
<td>6.87 (7.11)</td>
<td>22.82 (23.67)</td>
</tr>
</tbody>
</table>

a Calibrated as ginsenoside Rb2.
b The data was presented as average of three replicates (R.S.D.s <5%).

Fig. 7. Dendrogram resulting from average linkage between groups hierarchical cluster analysis. The hierarchical clustering was done by SPSS software. A method named as average linkage between groups was applied, and Squared Euclidean distance was selected as measurement. Dendrogram resulting from the contents of nine investigated saponins including notoginsenoside R1, ginsenoside Rb1, Rb2, Rb3, Rc, Rd, Re, Rf and Rg1. S1, S2 and S3: Pharmaceutical Factory A; S4, S5 and S6: Pharmaceutical Factory B; S7 and S8: Pharmaceutical Factory C; S9 and S10: Pharmaceutical Factory D.

4. Conclusion

The developed HPLC–ESI-MS/MS method is rapid and sensitive. It can be used for qualitative and quantitative determination of ginsenosides and notoginsenosides, which is helpful to improve the quality control of P. notoginseng and its pharmaceutical preparations such as XUESETONG injection.

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References