Available online at www.sciencedirect.com
SCIENCE DIRECT®
Talanta 66 (2005) 1005–1011
Talanta
www.elsevier.com/locate/talanta

Development of an indirect competitive ELISA for the determination of papaverine

Jin Yan[a], Jian-Qiu Mi[a], Jian-Tao He[a], Zhen-Quan Guo[b], Mei-Ping Zhao[a,*], Wen-Bao Chang[a]

[a] *College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, PR China*
[b] *College of Life Sciences, Peking University, Beijing 100871, PR China*

Received 16 July 2004; received in revised form 3 January 2005; accepted 4 January 2005
Available online 30 January 2005

Abstract

Papaverine (1-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinoline, PAP) is a member of the benzylisoquinoline sub-group of the opium alkaloids. It has been widely used for treating diseases like pulmonary arterial embolism and renal or biliary colic. In this paper, a specific conjugate of mono-demethylated papaverine-*O*-carboxylmethyl ether (MDMPAP-*O*-CME) and bovine serum albumin (BSA) was synthesized and used as the complete antigen (PAP-BSA), with which we successfully obtained a high-titer anti-PAP polyclonal antibody (pAb) by immunization of rabbits. The anti-PAP pAb showed high affinity to papaverine with an affinity constant (K_{aff}) of 7.3×10^7 L/mol. With this antibody, we established a sensitive immunochemical method for the determination of papaverine based on indirect competitive enzyme-linked immunosorbent assay (ELISA). The optimal concentrations of the coated antigen (PAP-OVA) and purified pAb used in the ELISA were 5 and 1.2 μg/mL, respectively. The cross reactivity of other benzylisoquinoline derived substances, including 1-(3,4-dihydroxybenzyl)-7-hydroxy-6-methoxy-isoquinoline (6-methoxy-papaveroline, MPAPO), morphine (MP) and codeine (CD) were all lower than 1%. The linear range of the calibration curve was 0.1–1000 ng/mL. Normal human serum samples were spiked with known amount of papaverine and measured by the ELISA. Recoveries were between 102% and 105%. Papaverine content in a commercial papaverine hydrochloride injection sample was also determined using the established ELISA. Compared with the results given by the control experiment of HPLC, the recoveries of ELISA to detect injection samples were 102–110%. The limits of detection for synthetic serum samples and injection samples of papaverine hydrochloride were 0.25 and 0.06 ng/mL, respectively.

Keywords: Papaverine(PAP); Enzyme-linked immunosorbent assay (ELISA); Polyclonal antibody (pAb)

1. Introduction

Papaverine, together with morphine, narcotine, codeine, thebaine and narceine, are the six opium alkaloids which occur naturally in the largest amounts. Papaverine was first isolated in opium in 1848 by Merck [1]. It presents in the extent of 0.5–1% in most varieties of opium. In 1909, Pictet and Gams accomplished the synthesis of papaverine and confirmed its molecular structure [1]. Since papaverine was effective for anti-spasm, it was soon applied to serve in cases when necessary to relax the blood vessel and to smooth the muscle. Despite its medical practice, it has undesirable side effects, such as causing prolonged fall in arterial blood pressure, when the intestinal tract is relaxed. Although papaverine is not under international narcotics control as the other members of opium alkaloids, such as morphine and codeine, its excessive use as medicament and the illegal use as food additive have attracted the interests of many researchers to explore the sensitive and reliable analytical methods for papaverine.

So far, numerous methods have been developed to isolate and determine the opium alkaloids in natural plants,

* Corresponding author. Tel.: +86 10 6275 8153; fax: +86 10 6275 1708.
E-mail address: mpzhao@pku.edu.cn (M.-P. Zhao).

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.01.001

biological samples and artificial products. Chromatographic and other techniques were fully established, including Thin layer chromatography (TLC)/High performance thin layer chromatography (HPTLC) [2,3], Gas liquid chromatography (GLC) [4], High performance liquid chromatography (HPLC) [5–9], Gas chromatography–mass spectrometry (GC–MS) [10], Capillary electrophoresis (CE) [11–13], electrochemistry [14], atomic spectrometer [15], spectrophotometry [16,17] and colorimetry [18]. TLC/HPTLC, GLC and HPLC techniques have long been used in drug-abuse screening programmes. TLC/HPTLC required minimal instrumentation, low cost, simple operation and less analysis time. But GLC and HPLC are superior in terms of precision and sensitivity. The limit of detection of HPLC can be as low as 40 ng/mL [7]. The disadvantages of GLC and HPLC are relatively high running costs and complicated instrumental operation. Due to the unique pattern of mass fragment intensities for each compound, LC/MS and GC/MS are often regarded as the most sophisticated or powerful qualitative tool currently in use; however, high demand of skilled operators and running costs limit their wide applications. Spectrophotometric methods are simple and applicable to a wide range of concentrations; however, they lack the specificity to distinguish between different opiate compounds.

Concerning of the biological activity of papaverine, we thought immunoassay method based on the highly specific interaction between the antigen and antibody could be a sensitive tool to detect papaverine. The only existing immunoassay method for papaverine was reported by Yeremenko et al. [19]. In their method, 3-amino papaverine was carboxylated so as to conjugate BSA to produce complete antigen. With the obtained anti-papaverine pAb, they developed a solid-phase enzyme immunoassay with the sensitivity of 1 μg/mL. In this paper, we synthesized a novel complete antigen for papaverine based on the mono-demethylation on the benzyl ring of papaverine and subsequent conjugation to BSA. The antigen was used to immunize the rabbits and produced a high-titer pAb against papaverine. With this pAb, we established a highly sensitive indirect competitive ELISA for the specific determination of papaverine.

2. Experimental

2.1. Apparatus

The ELISA results were measured with Tecan Genios Microplate Reader (Austria). The absorbance and reference wavelengths were set at 450 and 492 nm, respectively. Cary 1E UV–vis Spectrometer (Varian) was used to measure the absorbance of the protein solution.

HPLC analysis was performed on HP 1100 system (Agilent Techonologies) with C_{18} column (Dikma Techonologies Diamonsil, 5 μ, 150 mm × 4.6 mm). The mobile phase consisted of methanol–acetonitrile–H_2O–glacial acetic acid (34:47:19:0.05, v/v) with a flow rate of 1.0 mL/min. The detection wavelength of UVD was set at 254 nm and 20 μL sample loop was used.

2.2. Materials and reagents

All chemicals used in HPLC were of HPLC grade. Papaverine was purchased from Lancaster (England). Morphine (MP) and codeine (CD) were obtained from Qinghai Pharmaceuticals Co. Ltd. (China) and National Institute for the Control of Pharmaceutical and Biological Products (China), respectively. Other chemicals used in this study were of analytical reagent grade. Distilled demineralized water was used throughout.

The coating buffer was 50 mmol/L sodium carbonate-sodium bicarbonate (pH 9.6). The 0.01 mol/L phosphate buffered saline (PBS) contained 8.0 g of NaCl, 2.9 g of $Na_2HPO_4{\cdot}12H_2O$, 0.2 g of KH_2PO_4 and 0.2 g of KCl dissolved in 1 L distilled water (pH 7.2–7.4). The blocking buffer was prepared by dissolving 5 g of skim milk powder in 100 mL of 0.01 mol/L PBS. The washing solution consisted of 500 μL of Tween-20 in 1 L of 0.01 mol/L PBS. The substrate stock solution was prepared by dissolving 6 mg of 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma) in 100 mL of dimethyl sulfoxide (DMSO). The substrate buffer was 0.1 mol/L phosphate buffer (pH 6.0, PB).

2.3. Preparation of standard solutions and sample solutions

Standard solutions of papaverine and analogs used in the ELISA were prepared by dissolving 1 mg of papaverine, MP, CD, papaverinol and 6-methoxypapaveroline (MPAPO) in 1 mL of 0.01 mol/L PBS and serially diluting to 2000, 200, 20, 2, 0.2, 0.02 ng/mL, respectively. Papaverine standard solutions used in HPLC were diluted to 30, 20, 15, 12, 10, 8, 5, 2, 1, 0.5 and 0.1 μg/mL, accordingly.

Synthetic human serum samples were prepared by spiking 40-fold diluted normal human serum with known amount of 1 mg/mL of papaverine to final concentrations of 100, 20, 2 and 0.8 ng/mL, respectively.

Injection samples of papaverine hydrochloride (Jiangsu Heng Rui Pharmaceuticals Co. Ltd.) were diluted with water to different concentration levels and measured by the presented ELISA method and the reference HPLC method, respectively, for comparison.

2.4. Synthetic way of the complete antigen

First, the papaverine was mono-demethylated by refluxing in 40% HBr for 15 min according to a previously method [20] with minor revision. The mono-demethylated papaverine (MDMPAP) was then activated by bromoacetic acid to obtain mono-demethylated papaverine-*O*-carboxylmethyl ether (MDMPAP-*O*-CME). Finally, MDMPAP-*O*-CME was reacted with BSA using 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide·HCl (EDC) and *N*-hydroxysuccinimide (NHS)

as the activated reagent to obtain the complete antigen of PAP-BSA conjugate. The conjugation ratio of MDMPAP-*O*-CME with BSA was measured by Coomassie Brilliant Blue spectrophotometric method [21]. The conjugate of MDMPAP-*O*-CME with ovalbumin (PAP-OVA) was prepared in the same way as the PAP-BSA.

2.5. Production and purification of anti-PAP pAb

Three New Zealand white rabbits in good health weighing 2–3 kg were raised and immunized according to the regular methods with the dosages of 1, 0.5, 0.5 and 0.5 mg of PAP-BSA on days 0, 10, 20 and 30, respectively. The blood of the rabbits was collected through the jugular artery 10 days after the final immunization. The blood cells were removed by centrifuging to obtain the antiserum. The antiserum was purified according to a modified caprylic acid–saturated ammonium sulfate method [22].

2.6. ELISA procedure used for the determination of papaverine

Aliquots of 100 μL of coating solutions with certain amount of PAP-OVA were added to the wells and incubated for 16 h at 4 °C. Possible non-specific adsorption was prevented by performing a subsequent incubation with aliquots of 250 μL of the blocking buffer at 37 °C for 2 h. Then, dilutions of 100 μL of diluted antiserum or purified anti-PAP pAb solutions were pipetted into the coated wells. After incubating for 1 h at 37 °C, 100 μL of goat anti-rabbit IgG–HRP solution (1:1000 diluted with 0.01 mol/L PBS) was added and incubated again for 1 h at 37 °C. Subsequently, 100 μL of substrate solution (containing 30% H_2O_2:6 mg/mL TMB:PB = 1.5:10:1000) was added into the wells and the enzyme reaction was developed in darkness for 15 min at room temperature. The reaction was terminated by adding 50 μL of 2 mol/L H_2SO_4 to each well. The microtiter plates were sealed during every incubation procedures to avoid evaporation and washed three times after each incubation procedures with the washing solution. Before the enzyme reaction procedure, the microtiter plates were washed twice with distilled water in addition.

In the competitive ELISA, aliquots of 50 μL of anti-PAP pAb solutions at optimal concentration and 50 μL of the standard solutions of one of the competitive compounds were mixed and pipetted into the coated wells, instead of above mentioned step of adding 100 μL of diluted anti-PAP pAb solutions.

The same competitive ELISA procedure was also used for quantitative detection of papaverine in injection samples of papaverine hydrochloride and synthetic human serum samples. The added amount of all the samples was the same as above standard solutions (i.e. 50 μL). HPLC method was performed to control the accuracy of competitive ELISA in quantitative detection of papaverine in the injection samples.

3. Results and discussion

3.1. Design of the complete antigen

As a small molecule, papaverine was not able to initiate an immune response by itself. But it can be used as a hapten to be conjugated to a large carrier protein to form a complete antigen. An important principle for choosing the conjugated position of the hapten is to expose its characteristic group to the largest extent. As for the papaverine molecule, we thought the structure of 6,7-dimethoxyisoquinoline was the characteristic group, which should be preserved in the activation step. In Yeremenko's method [19], the activation on 3-isoquinolinamine and conjugation of carboxylated 3-amino papaverine to BSA caused inadequate exposure of the characteristic structure of papaverine. Thus, the sensitivity of the reported ELISA method was limited to 1 μg/mL. In our study, papaverine was first demethylated to MDMPAP so as to generate an active hydroxyl group on the benzyl ring, which had the least influence on the characteristic group of 6,7-dimethoxyisoquinoline. Then the phenolic hydroxyl of MDMPAP was carboxylated to introduce a carbon chain with carboxyl group on the end. Finally, the conjugation took place between the amino group of BSA and the carboxyl group of MDMPAP-*O*-CME (Scheme 1). The conjugation ratio of MDMPAP-*O*-CME with BSA was found to be 17:1. Using this complete antigen, an antiserum with a satisfactory titer as high as 1:50 000 (antiserum dilution corresponding to 50% of the maximum absorbance) was obtained.

3.2. Purification and characterization of the anti-PAP pAb

The protein concentrations of the antiserum and the purified pAb solution were determined by ultraviolet spectrometry and calculated based on the following equation [21]:

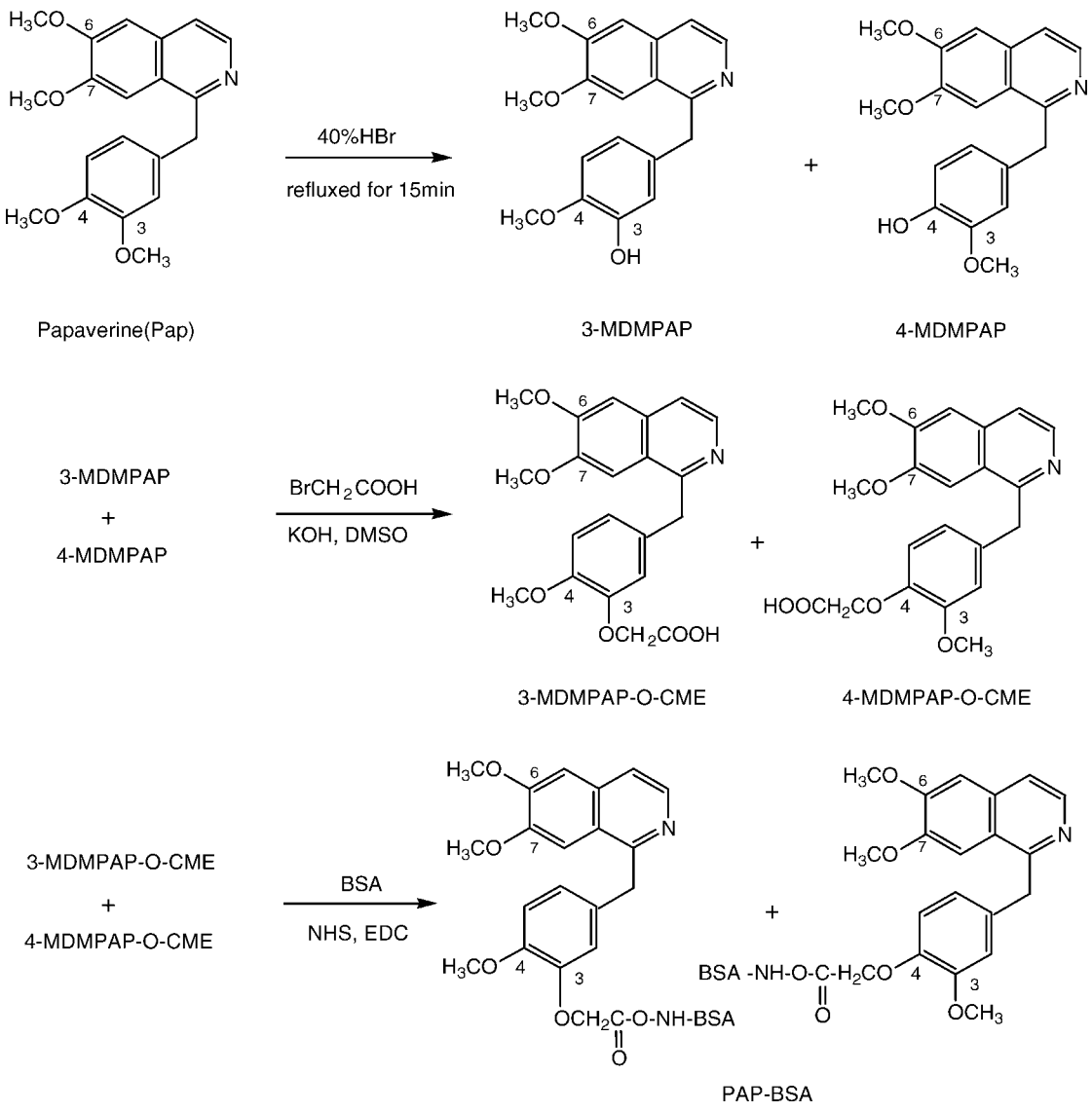
$$c_{\text{protein}}(\text{mg/mL}) = 1.45A_{280\,\text{nm}} - 0.74A_{260\,\text{nm}}$$

Affinity constant (K_{aff}) value represents the combined stability of Ag–Ab complex, which is a crucial parameter in estimating the quality of the anti-PAP pAb. K_{aff} could be determined by ELISA based on the Law of Mass Action [23]. The amount of anti-PAP pAb bound to the coated antigen was represented as a sigmoidal curve of absorbance (A) versus the logarithm of antibody concentration added to each well (Fig. 1).

K_{aff} could be calculated by the following formula [23]:

$$K_{\text{aff}} = \frac{n-1}{2(n[\text{Ab}']_t - [\text{Ab}]_t)}, \quad n = \frac{[\text{Ag}]_t}{[\text{Ag}']_t}$$

$[\text{Ag}]_t$ and $[\text{Ag}']_t$ are two different coated antigen concentrations, while $[\text{Ab}']_t$ and $[\text{Ab}]_t$ are the observed anti-PAP pAb concentrations at A-50 (50% of the maximum A value) corresponding to the plates coated with $[\text{Ag}']_t$ and $[\text{Ag}]_t$. The affinity constant of the anti-PAP pAb with PAP-OVA was found to be 7.3×10^7 L/mol, which was high enough for the later use



Scheme 1. Synthetic strategy of the complete antigen of papaverine.

in immunoassay method. Also from the serially diluting anti-PAP pAb curves at different coated antigen concentrations, the optimal concentrations of the coated antigen and the purified anti-PAP pAb for the ELISA use were determined to be 5 and 1.2 μg/mL, respectively.

3.3. *Specificity tests of anti-PAP pAb used in ELISA*

The cross-reactivity is an important parameter, when using ELISA method to detect real samples of opium and poppy plant. The alkaloids of the poppy plant are the same as those of opium. The relative proportions of the different alkaloids vary greatly in different kinds of opium and poppy plant, which is usually 8–19% of morphine, 1–4% of codeine, and 0.5–1% of papaverine. Because of their massive proportions, morphine and codeine were examined for the cross reactivity with the anti-PAP pAb, so as to prove the feasibility of determination of papaverine in real samples using ELISA method. The cross-reactivity of other structural analogs of papaverine, such as papaverinol and 6-methoxypapaveroline (MPAPO) was also tested (Fig. 2). The papaverinol and MPAPO used for the test were both synthesized by ourselves, according to the previous methods reported by Hermann et al. [24] and Brossi and Teitel [20].

It could be clearly seen from Fig. 3 that with the increase of the concentrations of the competitive compounds, the immunoreaction between the antibody and antigen was significantly inhibited by papaverinol, while the other three alkaloids had little influence on the reaction. The cross reactivity of papaverinol with papaverine was 31% based on the 50% displacement method [25]. As for the other three compounds of MPAPO, morphine and codeine, cross reactivities were all lower than 1%, which meant they would have little interference on the immunoassy of PAP. Regarding of the common part of the structures of PAP and papaverinol, it might be concluded by our experimental results that the structure

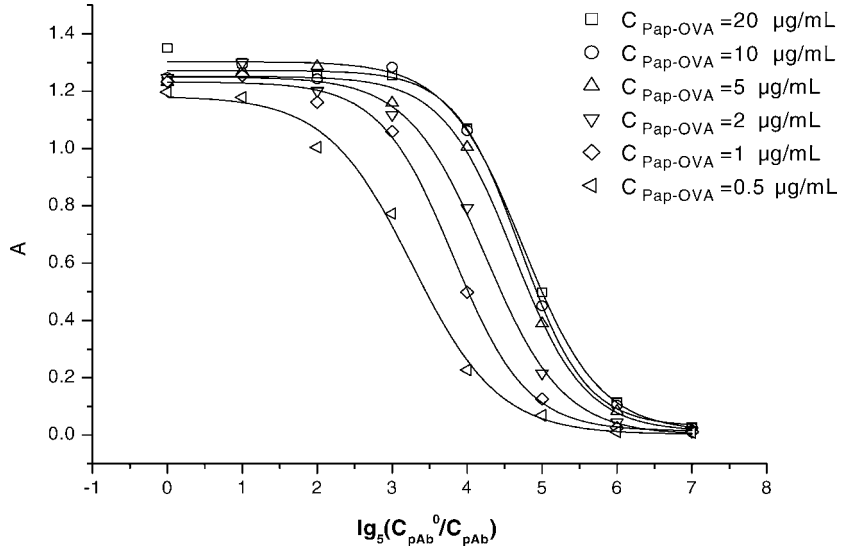


Fig. 1. Titration curves of serially diluted purified antibody at different concentration of coated antigen. The primary concentration of anti-PAP pAb (C^0_{pAb}) was 0.72 mg/mL. The concentrations of coated antigen (PAP-OVA) were 20, 10, 5, 2, 1 and 0.5 μg/mL (100 μL for each well). Purified pAb was five-folds serially diluted at each concentration of coated antigen.

of 6,7-dimethoxyisoquinoline was the particular and critical group that caused the specific discrimination of the anti-PAP pAb. And this was most probably the reason why our anti-PAP pAb showed better performance in the later application compared to Yeremenko's method.

3.4. Application of the ELISA to synthetic human serum samples and injection samples of papaverine hydrochloride

The calibration curve of the indirect competitive ELISA method was established based on logit–log algorithm. The linear range was found to be 0.1–1000 ng/mL with the regression equation of $\ln(A/(A_0 - A)) = 1.489 - 1.340 \log c_{PAP}$ ($R = 0.991$, $n = 5$). The limit of detection was found to be 0.06 ng/mL (defined as three times of the standard deviation of the blank).

When applying this method to human serum samples, 1:40 dilution of the serum samples with 0.01 mol/L PBS was necessary prior to addition to the well to prevent the matrix influence. The recovery results were shown in Table 1. The limit of detection was found to be 0.25 ng/mL for the synthetic human serum samples and the within-assay coefficient of variation was less than 13%.

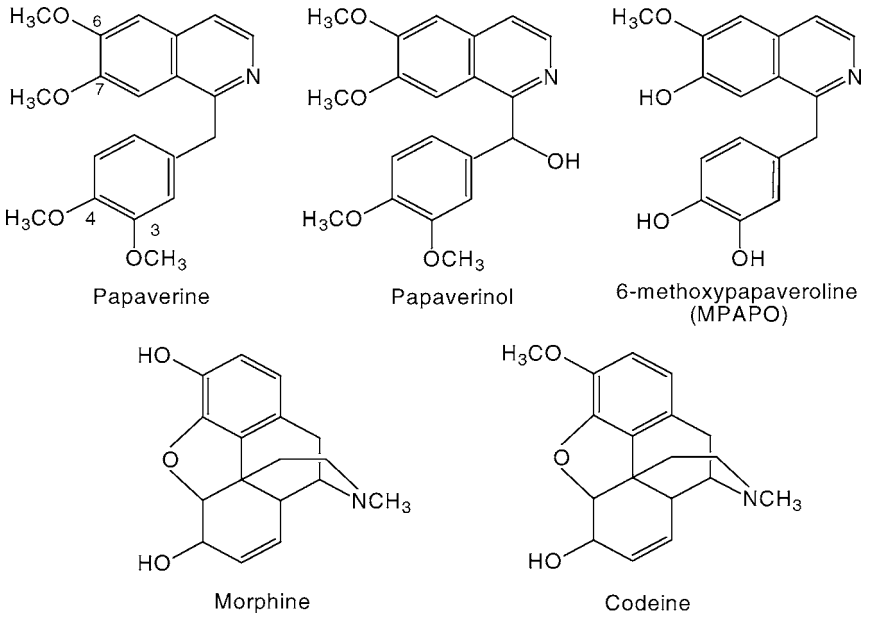


Fig. 2. Structures of the compounds used in the determination of cross reactivity.

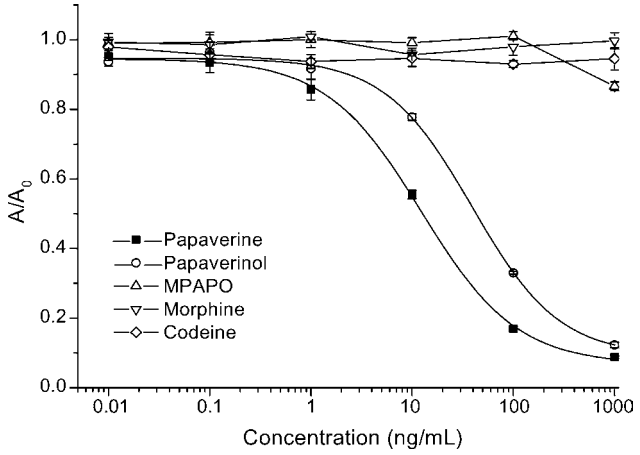


Fig. 3. Cross-reactivity test results of several benzylisoquinoline derivatives based on the competitive ELISA. The concentration of coated antigen of PAP-OVA was 5 μg/mL. The concentrations of the competitive compounds of papaverine, papaverinol, 6-methoxypapaveroline (MPAPO), morphine and codeine were 1000, 100, 10, 1, 0.1, 0.01 ng/mL in each well, respectively. The concentration of limited anti-PAP pAb was 1.2 μg/mL in each well.

Table 1
Recoveries of papaverine in synthetic human serum samples by the indirect competitive ELISA method

Spiked amount of PAP in each well (ng/well)	Recovery[a] (%)	R.S.D.[b] (%)
5	105	6
1	102	7
0.1	104	13
0.04	98.7	11

[a] The recovery was determined by spiking normal human serum samples with known amount of PAP and comparing with standard controls. The data was expressed as mean of six experiments.
[b] The R.S.D. result was obtained by six experiments.

No significant matrix influence was observed, when applying this method to injection samples of papaverine hydrochloride. Papaverine hydrochloride injections were diluted at ratios of $1{:}3 \times 10^5$, $1{:}1.5 \times 10^6$ and $1{:}1.5 \times 10^7$ to be determined by the ELISA method. HPLC method was performed as the control method to provide the reference concentration of the papaverine hydrochloride injection sample. The calibration equation for HPLC method was $A(\text{mAu}) = 23.35 + 162.49 c_{\text{PAP}}$ ($R = 0.9999$, $n = 3$), with the linear range of 0.1–30 μg/mL and limit of detection of 0.05 μg/mL (defined as three times of the S/N). The reference concentration of papaverine hydrochloride injection was 28.7 mg/mL as determined. In comparison with this value, the recovery results given by ELISA were between 102% and 110% (Table 2). The limit of detection for the papaverine hydrochloride injection samples by the ELISA method was found to be 0.06 ng/mL. The within-assay coefficients of variation were less than 2% and 13% for HPLC method and ELISA method, respectively.

Obviously, the established ELISA method is more sensitive than the existing chromatographic methods, whose detection limits for papaverine were 40 ng/mL (by HPLC) [7] and 0.4 ng/mL (by GC–MS) [10]. Furthermore, the merits of high specificity, simplicity, small sample amount and potentiality to be developed into portable test kits make the ELISA a very powerful tool to detecting papaverine at low concentration levels.

In Yeremenko's method, the antibody was immobilzed on the solid phase. Papaverine in the sample and the peroxidase-labeled papaverine were allowed to compete for the limited quantities of antibody. One drawback of this type of ELISA was partial loss of the Fab section of the antibody (fragment for antigen binding), which might have been used for adsorption on the microplate. This would lead to significant weakening of the binding ability of antigen–antibody. In our present ELISA, the adsorbing problem of the Fab section of the antibody was successfully resolved by substitution of coating PAP-OVA onto the plate, following with the step of papaverine in the sample under study competing with coated complete antigen for limited quantities of free antibodies. Thus, by employing a new strategy for the synthesis of the complete antigen and modification of the ELISA procedure, the developed ELISA achieved a great improvement in sensitivity compared to the existing method (limit of detection 1 μg/mL) [19].

In addition, the low cross reactivity with other opium alkaloids, including morphine and codeine, offered a unique determination method for papaverine in nature plant without interference.

Table 2
Recoveries of papaverine in the injection samples of papaverine hydrochloride by the indirect competitive ELISA method

Total diluting folds of PAP hydrochloride injection in each well	Measured concentration[a] (ng/mL)	R.S.D.[b] (%)	Calculated concentration of PAP hydrochloride injection[c] (mg/mL)	Recovery[d] (%)
6×10^5	52.8	10	31.7	110
3×10^6	9.9	12	29.8	104
3×10^7	0.98	8	29.4	102

[a] The measured concentration was the results determined by the established ELISA method. Each point was measured in six times.
[b] The R.S.D. result was obtained by six experiments.
[c] The calculated concentration of papaverine hydrochloride injection corresponds to the measured concentration by ELISA multiplied by the diluting folds.
[d] The recovery was determined by comparison of the ELISA results of the PAP hydrochloride injection with the reference results given by HPLC. The original concentration of PAP in the injection was found to be 28.7 mg/mL based on the HPLC analysis.

4. Conclusion

Considering its advantages of low cost, rapidness, sensitivity and no need for complex pre-treatment steps, the presented ELISA seems to be a very promising technique for a large scale of studies, such as survey on patients under treatment of papaverine hydrochloride, which is useful for monitoring the personal discrimination between treatments and guiding physicians to adjust the dosage for different individuals. Overall, it can be a practical alternative for monitoring papaverine in biological fluids and a number of other related samples.

Acknowledgements

This work was financially supported by the Ministry of Science and Technology of People's Republic of China under the "Tenth-Five" Key Project (No. 2001BA804A21).

References

[1] D. Elad, D. Ginsburg, Bull. Narcotics (UNDOC) 3 (1952) 27.
[2] A.S. Saushkina, T.I. Maksimenko, I.N. Klimenko, O.M. Shcherbina, Farm. Zh. 2 (1992) 41.
[3] Z.L. Li, X.H. Zhao, Y.B. Su, Y.Z. Chen, B.C. Zhang, Chin. J. Chromatogr. 8 (1990) 388.
[4] K.K. Kaistha, J. Pharm. Sci. 61 (1972) 655.
[5] N.R. Ayyangar, S.R. Bhide, J. Chromatogr. 436 (1988) 455.
[6] D.B. Borojević, D. Radulović, D. Ivanović, P. Ristić, J. Pharm. Biomed. 21 (1999) 15.
[7] H.Y. Hao, J.X. Guo, Q.S. Shun, J. Liang, Y.Q. Yu, Z.T. Che, Acta Pharm. Sin. 35 (2000) 289.
[8] D.V. Singh, S. Prajapati, S. Bajpai, R.K. Verma, M.M. Gupta, S. Kumar, J. Liq. Chrom. Rel. Technol. 23 (2000) 1757.
[9] N. Fabre, C. Claparols, S. Richelme, M.L. Angelin, I. Fouraste, C. Moulis, J. Chromatogr. A. 904 (2000) 35.
[10] B.D. Paul, C. Dreka, E.S. Knight, M.L. Smith, Planta Med. 62 (1996) 544.
[11] I. Bjørnsdottir, S.H. Hansen, J. Pharm. Biomed. 13 (1995) 1473.
[12] I. Bjørnsdottir, S.H. Hansen, J. Pharmaceut. Biomed. 13 (1995) 687.
[13] Z.L. Yu, Y. Chen, L.W. Chang, Chin. J. Anal. Chem. 24 (1996) 444.
[14] M.A. Zarechenskii, O.M. Gaidukevich, V.P. Georgievskii, Farm. Zh. 4 (1991) 65.
[15] M. Eisman, M. Gallego, M. Varcarcel, J. Pharm. Biomed. Anal. 12 (1994) 179.
[16] N.T. Abdel-Ghani, A.F. Shoukry, Y.M. Issa, O.A. Wahdan, J. Pharm. Biomed. 28 (2002) 373.
[17] Y. Tang, J. Luan, Q. Wang, L. Wu, R. Cao, S.H. Liu, Acta Acad. Med. Sin. 24 (2002) 413.
[18] D.K. An, Pharmaceutical Analysis, 3rd ed., People Health Press, Beijing, 1996, p. 160.
[19] S.N. Yeremenko, I.Ye. Sukhov, I. Ye, N.P. Danilova, O.N. Tolkachev, R.G. Vasilov, A.I. Miroshnikov, Khim. Farm. Zh. 26 (1992) 100.
[20] A. Brossi, S. Teitel, J. Org. Chem. 35 (1970) 1684.
[21] Y.W. Xu, Immunoassay Techniques, 2nd ed., Science Press, Beijing, 1997, p. 393.
[22] D. Tang, Y.C. Wang, W.B. Chang, Y.X. Ci, Chin. J. Anal. Chem. 27 (1999) 899.
[23] J.D. Beatty, B.G. Beatty, W.G. Vlahos, J. Immunol. Methods 100 (1987) 173.
[24] T.W. Hermann, U. Girreser, P. Michalski, K. Piotrowska, Arch. Pharm. Pharm. Med. Chem. 4 (2002) 167.
[25] J.J. Pratt, Clin. Chem. 24 (1978) 1869.