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Journal of Chromatography B, 861 (2008) 130–135
JOURNAL OF CHROMATOGRAPHY B
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Short communication

A rapid and validated HPLC method to quantify racecadotril metabolite, thiorphan, in human plasma using solid-phase extraction

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Received 21 September 2007; accepted 26 November 2007
Available online 4 December 2007

Abstract

A HPLC method with UV detection was developed and validated for the determination of thiorphan in human plasma. Nevirapine was used as the internal standard. Separation was performed by a Waters sunfire C_{18} reversed-phase column maintained at 35 °C. The mobile phase was a mixture of 0.05 M phosphate buffer with the pH adjusted to 2.6 and acetonitrile (74:26, v/v) at a flow rate of 1.0 mL/min. The UV detector was set at 210 nm. An original pre-treatment of plasma samples was developed, based on solid-phase extraction (SPE) with solid-phase extraction cartridges (Oasis HLB 3 mL, 60 mg). The extraction recovery for plasma samples of thiorphan at 0.1, 0.4 and 2.0 μg/mL was 93.5%, 98.2% and 97.8%, respectively. The calibration curve was linear with the correlation coefficient (r) above 0.9998. Linearity was verified over the range of 0.05–4 μg/mL thiorphan in plasma. The limit of quantification (LOQ) is 0.05 μg/mL. The mean accuracy was 92.7–99.6%. The coefficient of variation (precision) in the within- and between-batch was 2.2–8.4% and 4.1–8.1%, respectively. This method is simple, economical and specific, and has been used successfully in a pharmacokinetic study of thiorphan.

Keywords: Racecadotril; Thiorphan; Human plasma; High performance liquid chromatography; Solid-phase extraction

1. Introduction

Racecadotril (acetorphan), *N*-[(*R*,*S*)-3-acetylmercapto-2-benzylpropanoyl]-glycine, benzyl ester, is a specific inhibitor of enkephalinase, and therefore prolongs the antisecretory effect of the endogenous enkephalins [1]. Previous studies have demonstrated that racecadotril is a pro-drug. Orally administered racecadotril is rapidly hydrolyzed to the more potent enkephalinase inhibitor thiorphan, *N*-[(*R*,*S*)-1-oxo-2-mercaptomethyl-3-benzylpropanoyl]-glycine [1,2] (Fig. 1).

Unfortunately, to our best knowledge, no analytical method using HPLC has been reported for the determination of thiorphan in human plasma. Only a few analytical methods using HPLC have been reported for the determination of thiorphan in rat plasma [3,4]. However, these techniques are not suitable or have not been investigated for human samples and are not sensitive enough (the sensitivity is 0.6 μg/mL) for our purposes. Methods using LC/MS/MS detection have been reported [2]. However, this equipment is not available in all clinical laboratories due to its expensiveness.

The present paper describes the development and the validation of a rapid HPLC method with UV detection, which is simple and sensitive, requires less expensive instrumentation and is suitable for the measurement of thiorphan concentrations in human plasma used in pharmacokinetic studies. The developed method is based on the employment of an original solid-phase extraction (SPE) procedure for the plasma pre-treatment.

2. Experimental

2.1. Chemicals and reagents

Racecadotril and nevirapine (internal standard (IS)) were obtained from Venturepharm Service CRO Group (Beijing, China) and thiorphan from Bachem Company (Bubendorf, Switzerland). Acetonitrile and methanol of HPLC-grade were obtained from Merck (Darmstadt, Germany). Sodium metabisulfite, phosphoric acid and potassium dihydrogen phosphate all pure for analyses were purchased from Shanghai

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doi:10.1016/j.jchromb.2007.11.038

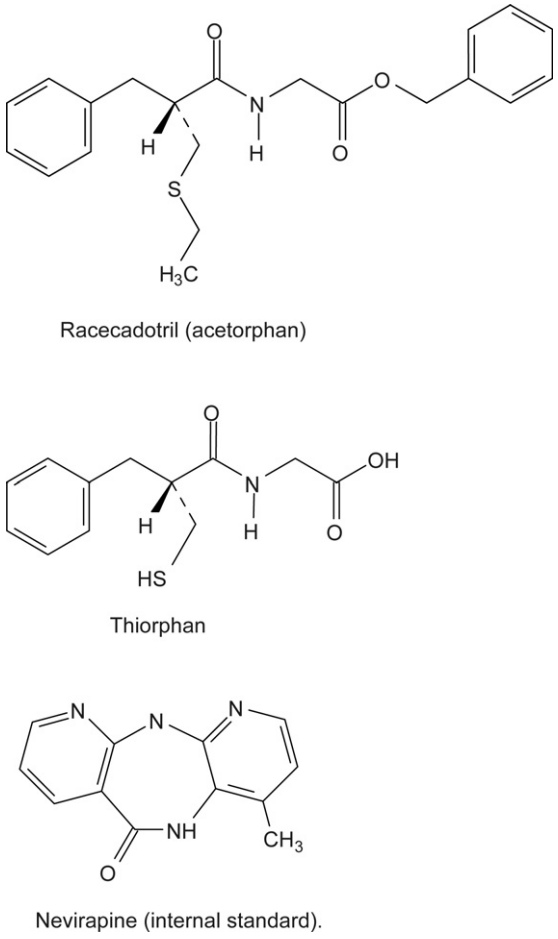

Fig. 1. Chemical structure of racecadotril, thiorphan and nevirapine (internal standard).

Chemical Reagent Company (Shanghai, China). Ultrapure water was obtained by means of a MilliQ apparatus by Millipore (Milford, USA). Blank plasma was provided by Kunming General Hospital of Chengdu Military Region (Kunming, China).

2.2. Instrumentation and chromatographic conditions

The chromatographic system was composed of a Waters 2695 separation module and a Waters 2487 dual wavelength detector (Waters, Corp., Milford, MA, USA). Detection and quantification were performed using Empower software. Separation was achieved with a Waters sunfire C_{18} reversed-phase column (150 mm × 4.6 mm, 5 μm) kept at 35 °C. The mobile phase was composed of a mixture of a 0.05 M phosphate buffer with the pH adjusted to 2.6 and acetonitrile (74:26, v/v). The flow rate was 1.0 mL/min. After spectrophotometric measurements (Fig. 2) the UV detector was set at 210 nm.

2.3. Preparation of stock and working solutions

Stock solution of thiorphan was prepared at concentration of 100 μg/mL in acetonitrile. Stock solution of IS was prepared at concentration of 200 μg/mL in methanol. Stock

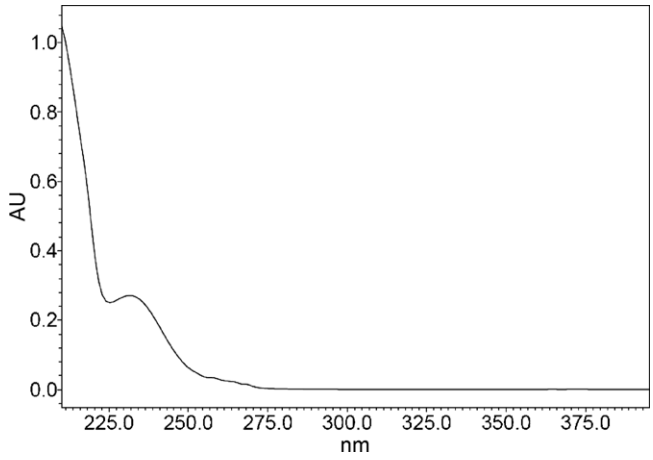

Fig. 2. UV spectrum of a 10 μg/mL thiorphan solution.

solutions were stored at −20 °C until they were used for preparing working solutions by adding the appropriate volume of water to prevent precipitation of proteins when added to the plasma. Working solutions of different concentrations were prepared from above-mentioned stock solutions afresh before to use.

2.4. Preparation of calibration standards and quality control (QC) samples

Calibration standards in plasma at concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2 and 4 μg/mL were prepared by spiking appropriate aliquots of working solutions of thiorphan. The QC samples were also prepared as above at concentrations of 0.1, 0.5, and 2 μg/mL, representing low, medium, and high concentration QC samples, respectively. These calibration standards and QC samples were prepared fresh for each analytical batch along with the unknown samples.

2.5. Sample processing

All plasma samples, to which antioxidant had been added, including blanks, standards, QC samples and unknowns, were extracted using solid-phase extraction cartridges (Oasis HLB 3 mL, 60 mg), which were purchased from Waters (Milford, MA, USA). Each cartridge was equilibrated by 1 mL of acetonitrile, and then conditioned by 1 mL of ultrapure water, and then conditioned by 1 mL of pH 2.4, 0.2 M phosphate buffer. One hundred microliter of 0.2 M phosphate buffer with the pH value adjusted to 2.0 and 100 μL of IS working solutions (4 μg/mL) were added into 500 μL plasma sample and vortex-mixed for 3 min. Thereafter, the mixed solution was loaded onto and passed through the cartridge without lab vacuum. After washed once with 1 mL of pH 2.4, 0.2 M phosphate buffer and once with 1 mL of ultrapure water, the analytes were then eluted with 1 mL of acetonitrile. The eluent was evaporated to dryness at 40 °C under a stream of nitrogen. The dried extract was then reconstituted with 100 μL of mobile phase, and a 20 μL was injected into the chromatographic system.

2.6. Validation of method

2.6.1. Calibration curve

The QC samples spiked with thiorphan at seven concentrations over the range of 0.05–4.0 μg/mL were prepared as described in Section 2.4. The calibration curves were constructed by plotting the peak height ratios of aimed compounds to IS versus the compound concentrations spiked.

The LOQ was defined as a reproducible lowest possible concentration, linear with the calibration curve having a coefficient of variation (CV) below 20% and accuracy between 80% and 120%. The LOQ was analyzed five times for conformation.

2.6.2. Accuracy and precision

Within-batch accuracy and precision evaluations were performed by analysis of samples consisting of calibration samples and five replicates of low, medium and high quality control samples for thiorphan as described in Section 2.4. Between-batch accuracy and precision were assessed by repeated analysis of thiorphan on three separate batches. The precision was defined as R.S.D. and the accuracy was assessed by comparing the measured concentration with its true value.

2.6.3. Extraction recovery

The extraction recovery of thiorphan at three different levels (low, medium and high) of 0.1, 0.4 and 2.0 μg/mL as well as IS at 4 μg/mL was evaluated by comparing the peak heights of five QC samples subjected to extraction to the mean peak heights of five unprocessed reference solutions, respectively.

2.6.4. Stability

Thiorphan stability in plasma was assessed by analyzing QC samples at concentrations of 0.1, 0.4 and 2.0 μg/mL, respectively, in triplicate ($n = 3$), for three freeze–thaws, short-term, long-term and post-preparative stabilities. For the short-term stability, the plasma samples were kept at room temperature (about 30 °C) for 4 h before sample preparation. The freeze–thaw stability of the thiorphan was determined over three freeze–thaw cycles within 3 days. In each freeze–thaw cycle, the spiked plasma samples were frozen for at −20 °C 24 h and thawed at room temperature. The long-term stability was evaluated after keeping the plasma samples frozen at −20 °C for 7 days. The stability of the prepared plasma samples was tested after keeping the samples in an autosampler at 20 °C for 4 h. The samples were analyzed and the results were compared with those obtained for the freshly prepared samples.

2.7. Pharmacokinetic study

The developed method was applied to investigate the concentrations of thiorphan in human plasma from healthy volunteers. Eighteen healthy male volunteers (19–28 years old, nonsmokers) were selected for the study. Before enrollment and at the end of the study, each volunteer underwent a physical examination and clinical laboratory tests, including bellows auscultation, palpation of liver and spleen, blood pressure, electrocardiogram, heart rate, liver function, and renal function, etc. After an overnight (more than 10 h) fast, volunteers received a single oral dose of 600 mg racecadotril granule (Venturepharm Service CRO Group, Beijing, China). Venous blood samples (4 mL) were collected before and at 0.08, 0.17, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6 and 8 h post-racecadotril dose. Each blood sample was stored in single-use heparinized glass tube (Insepack, China) containing fresh sodium metabisulfite saturated solution (50 μL), then centrifuged (within 1 h from collection) at 3200 × g for 5 min; the supernatant (plasma) was then transferred into polypropylene test tubes and stored at −20 °C until HPLC analysis.

3. Results and discussion

3.1. Separation

Fig. 3 shows the representative chromatograms of blank plasma, plasma sample spiked with 0.05 μg/mL (LOQ) of thiorphan and IS, and plasma sample obtained from a healthy volunteer 45 min after the administration of an oral 600 mg dose of racecadotril. The analyte separated well from IS under the described chromatographic conditions, at retention times of 5.7 and 9.6 min. No interference with constituents from the plasma matrix was observed. Furthermore, racecadotril was not detected at the retention time of 7.1 min in plasma sample from healthy volunteers after administration of an oral 600 mg dose of racecadotril.

3.2. Linearity, sensitivity and quantification limit of the assay

The peak height ratio of thiorphan to IS in human plasma was linear with respect to the analyte concentration over the

Table 1
Precision and accuracy of thiorphan calibration curve points in human plasma ($n = 5$)

Added concentration (μg/mL)	Measured concentration (mean ± S.D., μg/mL)	Accuracy (%)	Precision (R.S.D.%)
0.05	0.052 ± 0.006	4.4	10.6
0.1	0.104 ± 0.007	3.6	6.9
0.2	0.191 ± 0.012	−4.5	6.0
0.4	0.390 ± 0.028	−2.6	7.2
1	0.986 ± 0.053	−1.4	5.4
2	1.988 ± 0.098	−0.6	4.9
4	4.039 ± 0.222	1.0	5.5

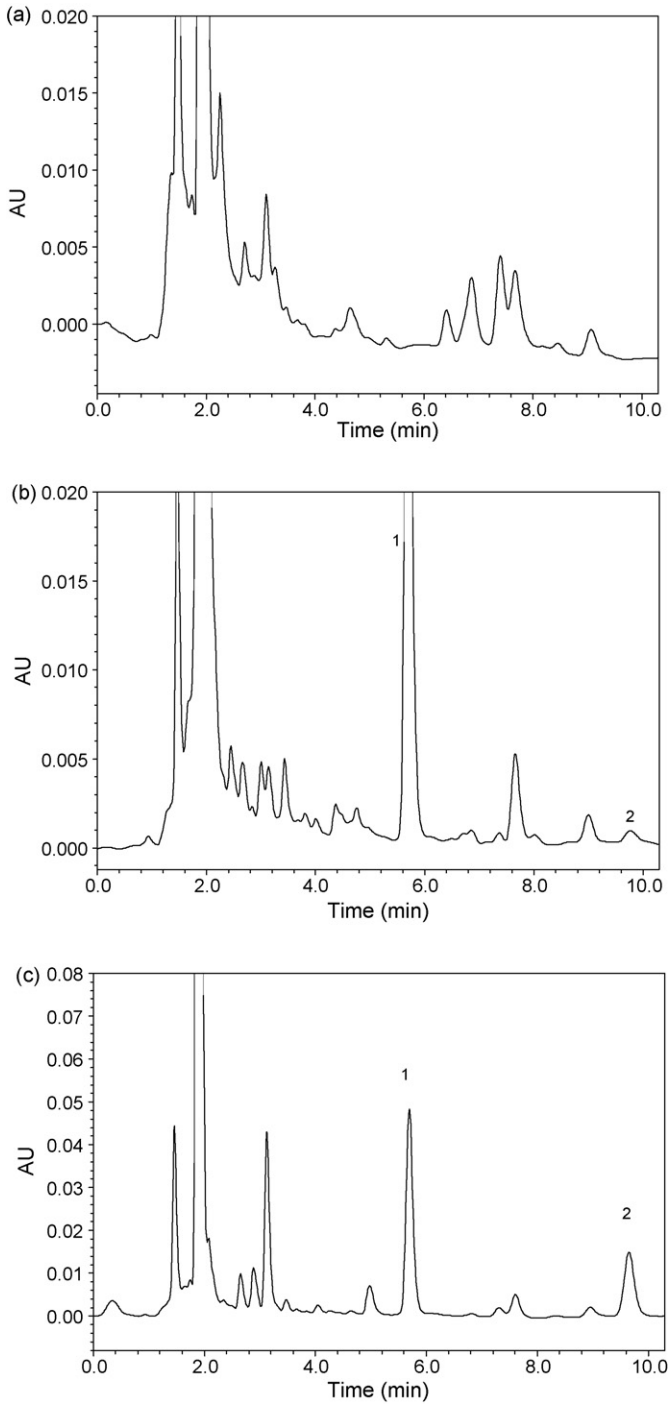

Fig. 3. Chromatograms of: (A) blank human plasma; (B) human plasma sample spiked with 0.05 μg/mL of thiorphan and IS; (C) plasma sample from a healthy volunteer 45 min after administration of a 600 mg oral dose of racecadotril. The plasma concentration was determined to be 1.062 μg/mL. Approximate retention times: thiorphan = 9.7 min; IS = 5.7 min. Peaks labelled 1 and 2 correspond to IS and thiorphan, respectively.

range of 0.05–4.0 μg/mL. The best fit for the calibration curve could be achieved with the linear equation $y = bx + a$ with a $1/x^2$ weighing factor. The mean linear regression equation of calibration curves for the analyte was $y = 0.276 \times -0.00107$ ($n = 5$), where y was the peak height ratio of the analyte to the IS and x was the concentration of the analyte in plasma. The correlation coefficient (r) for thiorphan was above 0.9998 over the concentration range used. Table 1 summarizes the calibration curve results for the analyte. These calibration curves were suitable for the generation of acceptable data for the concentrations of the analyte in the calibration samples during method validation and subject sample detection. The limit of quantification, defined as the concentration of thiorphan, was 0.05 μg/mL.

3.3. Specificity

Six randomly selected blank human plasma samples, collected from different healthy volunteers, were carried through the extraction procedure and chromatographed to determine whether endogenous plasma components interfere with the analyte or internal standard. No interfering peaks present at the retention time of either the analyte or IS.

3.4. Accuracy and precision

The accuracy values for between- and within-batch studies at low, medium and high concentrations of thiorphan in human plasma were within acceptable limits (Table 2). The results also indicated that the assay method was reproducible for replicate analysis of thiorphan within the same batch and on different batches.

3.5. Extraction recovery

The extraction recovery of thiorphan at low, medium and high quality control samples was 93.5 ± 6.4%, 98.2 ± 5.6% and 97.8 ± 2.4%, respectively. This indicates that extraction recovery of thiorphan is independent of concentration. The recovery of IS was 92.3% at the concentration used in the assay. Recovery data are summarized in Table 3.

3.6. Stability

Thiorphan was unstable and oxidated easily [1,5]. Thiorphan standard solution was stable after being placed at −20 °C for 2 months [5] and at 20 °C for 4 h. But it was stable after being placed at room temperature (about 30 °C) for only 0.5 h. The different concentration and volume of vitamin C, cysteine solution [2] and sodium metabisulfite saturated solution were tried as antioxidants. It was found that the sodium metabisulfite saturated solution (50 μL) as antioxidant added into human blood (4 mL) was the most effective to slow the rate of thiorphan oxidation.

In plasma, to which antioxidant had been added, thiorphan was stable after being placed at room temperature (about 30 °C) for 4 h and after undergoing three freeze–thaw cycles during frozen storage at −20 °C and after being stored in a freezer at −20 °C for at least 7 days. However, thiorphan will be unstable without antioxidant protecting after being extracted from plasma, so the prepared plasma sample was suggested to be

Table 2
The accuracy, within- and between-batch precision data for the measurement of thiorphan in human plasma

Added concentration (μg/mL)	Within-batch ($n=5$)			Between-batch ($n=3$)		
	Concentration found (mean ± S.D., μg/mL)	Precision (R.S.D.%)	Accuracy (%)	Concentration found (mean ± S.D., μg/mL)	Precision (R.S.D.%)	Accuracy (%)
0.1	0.097 ± 0.008	8.4	−3.0	0.097 ± 0.008	8.1	−3.0
0.4	0.371 ± 0.023	6.2	−7.3	0.384 ± 0.021	5.5	−4.0
2	1.931 ± 0.042	2.2	−3.5	1.992 ± 0.083	4.1	−0.4

Table 3
Recoveries of thiorphan and IS ($n=5$)

Compound	Concentration (μg/mL)	Recovery (%) (mean ± S.D.)	R.S.D. (%)
Thiorphan	0.1	93.5 ± 6.4	6.8
	0.4	98.2 ± 5.6	5.7
	2	97.8 ± 2.4	2.4
IS	4	92.3 ± 1.6	1.1

Table 4
Stability of Thiorphan at different conditions ($n=3$)

Sample concentration (μg/mL)	Concentration found (mean ± S.D., μg/mL)	Recovery (%)	Precision (R.S.D.%)
Short-term stability (30 °C, 4 h)			
0.1	0.097 ± 0.006	97.0	7.7
0.4	0.375 ± 0.017	93.8	6.6
2	1.866 ± 0.101	93.3	7.0
Long-term stability (−20 °C, 7 days)			
0.1	0.103 ± 0.009	103.0	8.8
0.4	0.386 ± 0.013	96.4	3.3
2	1.987 ± 0.046	99.4	2.3
Three freeze and thaw cycles			
0.1	0.101 ± 0.006	101.3	6.1
0.4	0.385 ± 0.031	96.1	8.0
2	2.114 ± 0.050	107.2	2.3
Post-preparative stability (20 °C, 4 h)			
0.1	0.106 ± 0.003	106.0	3.7
0.4	0.386 ± 0.004	96.5	2.0
2	2.075 ± 0.031	103.8	1.2

detected within 4 h at 20 °C. Stability data are summarized in Table 4.

3.7. Application to clinical study

The method was applied to determine the plasma concentrations of thiorphan after oral administration of 200 mg racecadotril to 18 volunteers. The mean plasma concentration–time curve of thiorphan is shown in Fig. 4. The PK parameters were received as follows: the maximum plasma concentration(C_{max}) 1.318 ± 0.254 μg/mL; area under the curve ($AUC_{0–8}$) 3.489 ± 0.465 μg h/mL; area under the curve ($AUC_{0–\infty}$) 3.879 ± 0.573 μg h/mL; the time to maximum plasma concentration (T_{max}) 0.842 ± 0.254 h; half-life ($t_{1/2}$) 2.67 ± 2.239 h. The biological $t_{1/2}$ of thiorphan activity is 3 h in the literature [1]. It is close to the result reported in our study. The biological half-life of thiorphan activity is 6.1 h in the literature [2]. It is different from the result reported in our study.

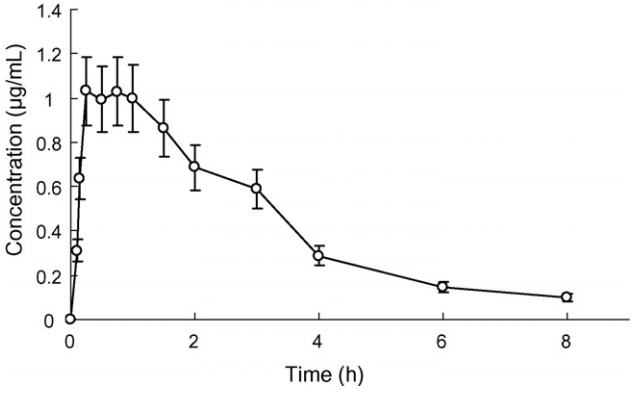

Fig. 4. Mean plasma concentration–time curve for thiorphan after an oral administration of 600 mg racecadotril to 18 healthy volunteers ($n=18$, mean value and S.D. are plotted).

From the concentration–time curve published in the literature [2] (Fig. 4), it is evident that the elimination of thiorphan is biphasic. Xu et al. followed the pharmacokinetics up to 24 h and they reported elimination half-life was 6.1 h. In our study, the pharmacokinetics was followed only up to 8 h and this is the reason of the short elimination half-life received (2.7 h). Although we had monitored the pharmacokinetics up to 12 h in this study, few thiorphan signals were detected out of 8 h because of the limitation of the sensitivity of our method. So this simple method is suitable for the pharmacokinetic studies of racecadotril after a dose above 600 mg. In order to study pharmacokinetics of racecadotril after lower doses (100–200 mg), it should be used three times or more of the plasma for sample extraction.

4. Conclusion

In this study, a sensitive and specific HPLC–UV method for the determination of thiorphan in human plasma was developed and validated. The SPE method using Oasis HLB cartridges achieved reproducible and high extraction recoveries of thiorphan and the IS (nevirapine). Compared with the HPLC reported methods [3,4], the method has the advantages of higher sensitivity, extraction recovery and broad linear range. The LC/MS/MS method described by Xu et al. [2], had better sensitivity (9.38 ng/mL), however, it requires more expensive instrumentation. The proposed method has been successfully applied to the pharmacokinetic studies of racecadotril metabolite-thiorphan in healthy male volunteers after oral administration of racecadotril. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instrument of LC–MS.

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