Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 10, pp. 1045–1047, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

Short Communication

HPLC analysis of mildronate and its analogues in plasma

O. SAHARTOVA, V. SHATZ* and I. KALVINŠ

Institute of Organic Synthesis, 21 Aizkraukles str., LV1006, Riga, Latvia

Keywords: *Mildronate; betaines; HPLC* p-*bromophenacyl derivatization; plasma analysis.*

Introduction

Mildronate (3-(2,2,2-trimethylhydrazinium)-propionate dihydrate) is used in cardiology and also as an adaptogene. It is an analogue of biologically important betaines: γ-butyrobetaine and carnitine (Fig. 1). Due to its ionic properties, mildronate and its analogues produce asymmetric peaks that are difficult to quantitate in normal-phase, reversed-phase and ion-pair chromatography. Therefore a novel system had to be found.

Another problem was low UV-absorbance of the solutes that prevented their direct detection in blood plasma samples. Thus, derivatization had to be used to achieve the desired sensitivity of analysis. It was shown that common methods of carboxylic acid derivatization were inefficient for betaines due to their relatively low reactivity. Therefore some special version of the procedure had to be developed.

In the present paper, an HPLC method of mildronate determination in blood plasma is described.

Experimental

Materials

Mildronate (quality according to the temporary pharmacopeal monograph) was obtained from Grindex (Riga, Latvia) γ-butyrobetaine and carnitine from Fluka (Switzerland). Acetonitrile used was of HPLC grade produced either by Cryochrom (St Petersburg, Russia) or by Reachim (Harkov, Ukraine). The distilled water was further purified from ionic and organic impurities with a Milli-Q system from Millipore (France). 18-crown-6 (purity >97%) was from Fluka (Switzerland). The salts of analytical grade used for buffer preparation were purchased from Reachim (Moscow, Russia).

Instrumentation

The HPLC system consisted of the following units from Gilson, France: a model 302 pump, model 811 dynamic mixer, model 803C manometric module, model 115 variable wavelength detector, 620/704 HPLC System Manager (Version 3.0). The samples were

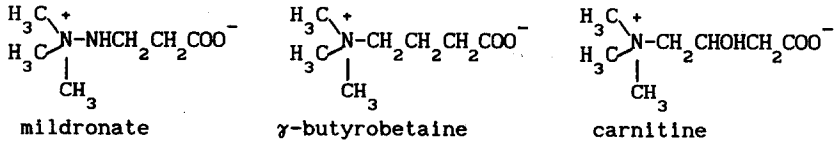

Figure 1
The compounds under study.

*Author to whom correspondence should be addressed.

injected via a 7125 injection valve, Rheodyne (CA, USA).

The column (4.6 × 150 mm) was packed with Silasorb 600 silica (Lachema, Czechoslovakia) by Elsico (Moscow). The eluent consisted of acetonitrile–0.025 M phosphate buffer (pH 5.7) (10:90, v/v). The buffer was prepared by dissolution of sodium dihydrophosphate (0.8 g) and potassium hydrophosphate (2.8 g) in deionized water (1.0 l), followed by pH adjustment with small amounts of KOH or H_3PO_4. After the addition of the required amount of acetonitrile, the solvent was degassed under vacuum and used at a flow rate of 1.5 ml min^{-1}.

Preparation of samples

Blood plasma samples were prepared as follows. A 400 mg mass of Dowex 50WX10 resin (20–50 mesh) were placed in a plastic column (8 mm internal diameter) and flushed with 1% of trifluoroacetic acid until the eluate pH reached 2–3, then with 2 ml of water. A 1 ml volume of deproteinized plasma was filtered through the prepared column. The filtrate was neutralized with 10 μl of 25% ammonium hydroxide, the excess of ammonia removed by nitrogen stream until mixture pH reached 6–8. The catalyst solution was prepared from 600 μl of 18-crown-6 solution (1 mg ml^{-1} in MeCN), 150 μl of potassium bicarbonate (0.1 mg ml^{-1} in water) and 150 μl of potassium hydrogen phosphate (0.15 mg ml^{-1} in water). The purified plasma sample (100 μl) was mixed with 450 μl of *p*-bromophenacyl bromide solution (5 mg ml^{-1} in MeCN) and 75 μl of catalyst solution in an amber glass vial. The vial was heated for 2 h at 70°C, and then the solvents evaporated under vacuum. The residue was dissolved in 400 μl of water, the solid impurities were removed by centrifugation. The solution (50–100 μl) was used for HPLC analysis.

Preparation of calibration graphs

In the experiments on external standard quantitation the stock solutions of mildronate, γ-butyrobetaine and carnitine (10 mg ml^{-1}) in water were prepared. Aliquots of these solutions were dissolved in 1 ml of deproteinized blood plasma to produce calibration solutions containing 5–100 μg ml^{-1} of the compounds under study. The samples were processed as described. The peak areas were used to construct the calibration graphs. When mildronate was determined using an internal standard method, γ-butyrobetaine was added to the sample to give a 20 μg ml^{-1} concentration.

Results and Discussion

The usual method of derivatization of acids with *p*-bromophenacyl bromide [1] was not applicable to betaines. It has been shown that in the determination of betaines in plant extracts the reaction could be catalysed by 18-crown-6 [2].

It was found that the presence of crown catalyst itself was not sufficient for the derivatization of betaines in plasma. The probable reason is that inorganic cations and some other components of blood plasma compete with mildronate for the active site of crown ether. The preliminary filtration of the sample through Dowex 50WX10 cation exchanger allowed the reaction of betaine derivatization to be completed in 2 h. The recovery of mildronate from the Dowex column was usually between 85 and 90%.

Carnitine and γ-butyrobetaine were tested as potential internal standards when reacted with *p*-bromophenacyl bromide under the described conditions.

The derivatization products have the properties of quaternary nitrogen bases and, therefore, they produce asymmetric peaks in reversed-phase mode. The other problem was rather poor separation of the betaine derivatives from the sample matrix peaks both in the traditional reversed-phase and in the ion-pair chromatography.

It is known [3, 4] that sometimes peak shape of bases obtained on unmodified silica columns with aqueous buffers as mobile phases is much better than that on reversed phases. This approach turned out to be efficient in the case of these compounds. The peak shape was quite satisfactory. Another advantage was that matrix peaks were eluted much closer to the dead volume and did not interfere the peaks of mildronate and its analogues.

Figure 2 shows a typical chromatogram of blood plasma spiked with 20 μg ml^{-1} of mildronate, γ-butyrobetaine and carnitine. The column was stable under the described conditions and its efficiency did not decrease considerably during 1 month of daily work.

The method of external standard can be used for the determination of the three substances.

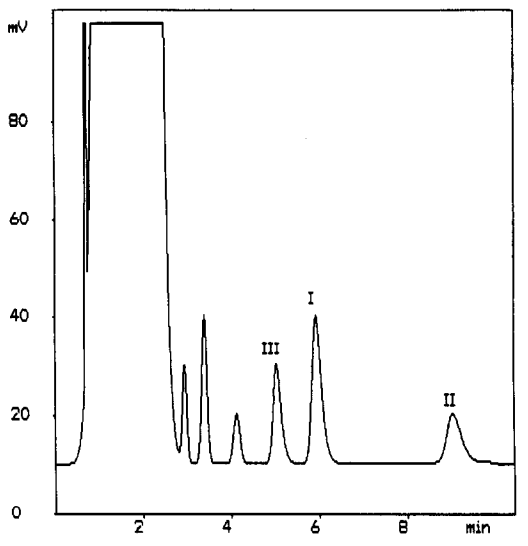

Figure 2
The chromatogram of blood plasma spiked with 20 μg ml^{-1} of mildronate (I), γ-butyrobetaine (II) and carnitine (III). Column: Silasorb 600, 4.6 × 150 mm; eluent: acetonitrile–0.025 M phosphate buffer pH 5.7 (10:90, v/v); detector: UV (λ = 262 nm).

The limits of quantitation for mildronate, γ-butyrobetaine and carnitine were 1.0, 2.5 and 0.75 μg ml^{-1}, respectively. The calibration graphs were linear in the range 5–100 μg ml^{-1} for plasma with relative standard deviations at the 20 μg ml^{-1} level of 6, 8 and 8% for mildronate, γ-butyrobetaine and carnitine, respectively.

In order to compensate the possible fluctuations of mildronate recovery from the ion-exchange column and derivatization reaction yield internal standard method can be used. γ-Butyrobetaine is the most convenient compound to be used for this purpose. The calibration relationship is expressed by equation

$$\frac{S_{\text{mildronate}}}{S_{\gamma\text{-butyrobetaine}}} = 0.13 + 1.381\,\frac{C_{\text{mildronate}}}{C_{\gamma\text{-butyrobetaine}}}.$$

The relative standard deviation of mildronate concentration for 5 and 20 μg ml^{-1} levels in this case was 4.5 and 3.8%, respectively.

References

[1] P.E. Minkler, S.T. Ingalls, L.S. Kormos, D.E. Weir and C.L. Hoppel, *J. Chromatogr.* **336**, 271–283 (1984).
[2] J. Gorham, E. McDonnell and R.G. Wyn Jones, *Analytica Chimica Acta* **138**, 277–283 (1982).
[3] B.A. Bidlingmeyer, J.K. Del Rios and J. Korpl, *Anal. Chem.* **54**, 442–447 (1982).
[4] R.M. Smith, T.G. Hurdley, R. Gill and D. Osselton, *J. Chromatogr.* **398**, 73–87 (1987).

[Received for review 7 May 1992; revised manuscript received 1 March 1993]