Journal of Chromatography, 502 (1990) 423–430
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 098

Note

Improved automated precolumn derivatization reaction of fatty acids with bromomethylmethoxycoumarin as label

J. H. WOLF and J. KORF*
Department of Biological Psychiatry, University of Groningen, Oostersingel 59, 9713 EZ Groningen (The Netherlands)
(Received August 2nd, 1989)

Derivatization and automation in high-performance liquid chromatographic (HPLC) analysis is very profitable for biomedical and clinical purposes, because of the gain in sensitivity and selectivity of the derivatization combined with a higher sample throughput. The most often used automated precolumn derivatization procedure is the analysis of amino acids and biogenic amines with *o*-phthalaldehyde[1]. The automation of this procedure is relatively simple as the agents and reagents are predominantly water soluble and the reaction kinetics are fast at room temperature.

Difficulties encountered by the automation of precolumn derivatization procedures in HPLC may include the use of elevated temperatures, the addition of two reagents at different time intervals, the addition of solid compounds, solvent extraction and the use of organic solvents as reaction media, which may result in peak distortion after direct injection in reversed-phase (RP)-HPLC[2].

In the HPLC of acidic compounds, bromomethylmethoxycoumarin (BrMMC) and its analogues have been extensively used as labels for derivatization[3–22]. Recently, we published a general method for the automated precolumn derivatization of fatty acids in serum and rat brain homogenates[23] for labels with a bromomethyl group, including BrMMC. In the manual procedure the label in coupled to the acid in an aprotic medium such as acetone or acetonitrile and this coupling is catalysed by the addition of solid potassium carbonate at 60°C. For the automation the following modifications were explored (see also ref. 23): lowering of the reaction temperature, replacement of the solid potassium carbonate and injection of a pure organic solvent. A satisfactory procedure was achieved by using a suspension of potassium carbonate in a solution of a crown ether and relatively high reagent concentrations. Peak distortion was reduced with a minimum decrease in peak height by using acetonitrile as solvent and acetonitrile–water mixtures as the mobile phase.

In our previous study the emphasis was more on the principle of the automation and solutions found for the indicated problems. The equipment used there had some drawbacks such as high blanks and clotting of the tubing by the suspension. With the use of a newer type of autosampler, the technical problems were solved. With the current modifications a very versatile system is obtained. Another disadvantage of our previous procedure was the formation of double peaks for every fatty acid.

0021-9673/90/$03.50

Investigations were made on the origin of this double labelling. In this paper we present an improved automated derivatization procedure for acidic compounds with BrMMC as label. The system can be used for a greater variety of reactions. As an example of the current procedure in biomedical research, we report the determination of fatty acids derived from small samples of rat blood taken during a stress procedure. The usefulness of this improved method has also been shown in drug studies[24,25].

EXPERIMENTAL

Reagents and chemicals

Analytical-reagent grade solvents were obtained from either Merck (Darmstadt, F.R.G.) or Baker (Deventer, The Netherlands) and were used without further purification except that methanol and acetonitrile were distilled. 4-Bromomethyl-7-methoxycoumarin, potassium carbonate (anhydrous) and 18-crown-6 were purchased from Aldrich (Beerse, Belgium) and the fatty acids from Sigma (St. Louis, MO, U.S.A.). 4-Bromomethyl-7-methoxycoumarin was dissolved in acetonitrile to give a working solution of 1 mg/ml. The suspension was prepared as follows: to 5 ml of a 20 m*M* solution of 18-crown-6 in acetonitrile were added 100 mg of potassium carbonte and 50 μl of water. After sonification for 30 min, the mixture was diluted with 5 ml of acetonitrile; the supernatant was separated from the solid potassium carbonate on the bottom of the vial.

Autosampler

The PROMIS autosampler (Spark, Emmen, The Netherlands) is equipped with a digital dispenser system, a miniaturized "finger pump". A coaxial pair of needles is inserted into the sample vial. Nitrogen pressure applied through the outer needle ensures that no air or vapour bubbles are formed during sample withdrawal. In our setup we used a commercially available two-way dispenser system, one line for the sample–suspension mixture and the other for the BrMMC solution. The coaxial needle is extended to approximately 1 mm above the hole in the side-port injection needle so that the needle is sufficiently under the liquid surface to ensure thorough mixing of the sample–suspension solvent.

HPLC equipment

Two Kratos SF400 pumps and a Kratos SF450 gradient mixer were used for solvent delivery. The samples were detected by a Kratos SF980 fluorescence detector equipped with a 5-μl cell, using an excitation wavelength of 325 nm and a cut-off filter of 398 nm. Data were collected with either a Kipp & Zonen (Delft, The Netherlands) BD8 recorder or an LDC CI-10 (Interscience, Breda, The Netherlands) integrator. The labelled fatty acids were separated on a Chrompack (Middelburg, The Netherlands) Chromspher C_{18} column (200 × 3 mm I.D.). The sample loop had a volume of 20 μl. All the chromatographic studies were performed at ambient temperature. A gradient of acetonitrile–water (80:20, v/v) to acetonitrile–methanol (50:50, v/v) in 45 min was used. Phosphoric acid was added to a final concentration of 25 m*M*. Elution was performed at 0.5 ml/min.

Blood samples

To 20 μl of rat blood 1 ml of acetonitrile containing margaric acid as internal standard was added. The precipitated protein was centrifuged. The supernatant is mixed 1:1 with the suspension in the vial of the autosampler.

RESULT AND DISCUSSION

Automation

In the published procedure[23], the reagents were delivered through a peristaltic pump, a standard option of the Spark SPH 125 PCD autosampler. The suspension is first mixed with the sample and subsequently with the BrMMC solution. Although all the analytical requirements were fulfilled, the system has some technical drawbacks. The choice of the tubing for the peristaltic pump is important. Acidic components from the plasticizers of the tubing can easily result in high blanks, whereas other tubing material slowly dissolves in the organic solvents. Clotting of the tubing by the suspension sometimes occurs.

In view of these problems, experiments were carried out with the newer PROMIS type of autosampler. This autosampler makes use of a dispenser to deliver the sample from the sample vial to the injection loop. Fig. 1 shows the original design (left) and the modified set-up used in this study (right). A double channel dispenser is used for adding the reagent BrMMC to the contents of the sample vial. In the sample vial the acid to be derivatized is added to the suspension. The outer needle is extended under the liquid surface. During sample withdrawal nitrogen is led through the outer needle, which ensures continuous agitation of the suspension, providing thorough mixing of the suspension–sample with the label. The dispenser is accurate and allows 20–50 μl out of a sample volume of 80 μl to be injected. With this system we determined valproic acid in human serum, rat blood and rat brain homogenates[24,25].

Investigations of the nature of the double labelling

In the automated reaction of fatty acids with BrMMC and the suspension every fatty acid showed two peaks (Fig. 2). The double labelling may be used for positive identification of the acids. Occasionally, depending on the state of the separation column, myristic acid and palmitoleic acid are not separated, but their second peaks are always separated. Accordingly, we use the second peak for the calculations. The longer the chain length, the larger is the second peak compared with the first (Fig. 2). The concentration of BrMMC does not influence the ratio for each doublet, whereas an increase in fatty acid concentration results in a relative decrease in the formation of the second peak.

No reports of double labelling were found in the literature. Therefore, we investigated whether other published procedures result in double labelling. For this, myristic acid was reacted with BrMMC under various conditions. Using our suspension[23], a ratio for the first to the second peak of 3.32 was found, whereas the frequently used manual procedure of Lam and Grushka[26] gave a ratio of 3.48. When the first peaks were compared with the internal standard, ratios of 0.61 and 0.58 were found. In their procedure, Lam and Grushka used a slight molar excess of 10% of BrMMC over the fatty acid. In our system, a much higher concentration of BrMMC is used to perform the reaction at room temperature. To establish the influenc of the

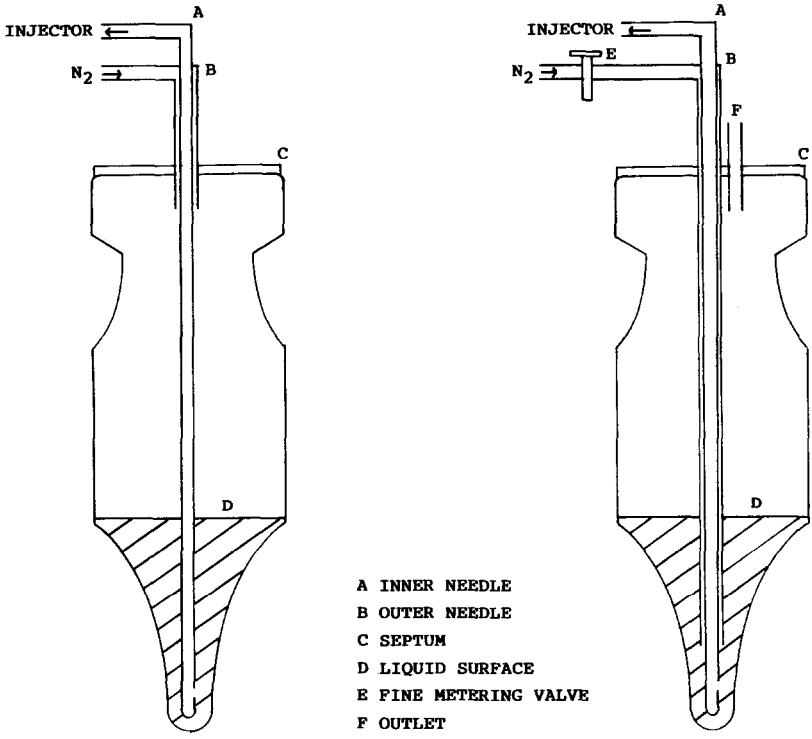

Fig. 1. (Left) the original and (right) the modified injection systems. Through the outer needle (B) nitrogen regulated with a fine metering valve (E) is led through the liquid. The extra outlet (F) prevents over-pressure.

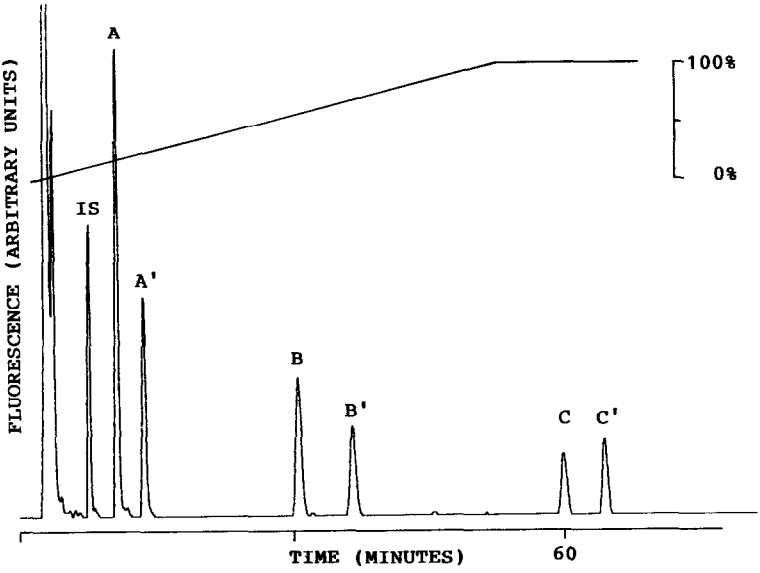

Fig. 2. Typical example of the double labelling found with BrMMC. Peaks: A = decanoic acid; B = myristic acid; C = stearic acid; IS = anthracene (internal standard). For separation conditions, see Experimental.

suspension with a high BrMMC concentration, solid potassium carbonate was used to replace the suspension. Now a ratio of 4.11 was found, so that these changes cannot explain the double labelling. Also, replacement of the suspension by tetraethylamine[13,18] gave similar results. The BrMMC concentration in the experiments was kept constant and a reaction temperature of 60°C and a reaction time of 30 min were used for the literature experiments. We conclude that the double labelling is not specific for our procedure.

In the automated procedure, the reaction mixture is injected directly onto the column, whereas in the non-automated procedure the reaction mixture is normally diluted with water to lower the solvent strengths to a composition compatible with the mobile phase. To establish whether direct injection is connected with the formation of the double labelling, the reaction mixture was diluted with water to the same composition as the mobile phase composition. Repetitive injection of this mixture for 24 h resulted in a slight decrease in the peak ratio within 3 h after the addition of the water. This reduction is due to a decrease in the second peak. The ratio of the first peak to internal standard was constant over a period of 24 h whereas the ratio of the second peak to internal standard decreased.

Three explanations can be given for the production of the double peaks: (a) impurity Y in the BrMMC reacts with fatty acid, FA, to form a product FA–Y; (b) FA–MMC, the methylmethoxy ester of a fatty acid, reacts with BrMMC via, for intance, an enol-keto tautomerization to form product FA–MMC–MMC or a structurally similar compound; (c) FA–MMC reacts under the influence of the base to give a product FA–X. By measuring the reaction constants, a discrimination between b on the one hand and a and c on the other can be made; reaction b is of second order whereas the other are of first order.

The kinetics of the derivatization reaction were investigated as a function of BrMMC concentration. The apparent rate of the reaction was evaluated by a simplex-optimized non-linear, least-squares curve fitting of first-order reaction kinetics to the time course data. The formation of the derivatives was first order with respect to BrMMC in both instances ([FA] = 3 pmol/μl; correlation coefficients >0.98).

As the rate of formation is first order with respect to BrMMC for both reactions, the second peak is not likely to be a degradative by-product of the primary reaction. Therefore, the second peak is produced in a competitive reaction with an impurity in the BrMMC. If the double labelling is caused by an impurity in the BrMMC, two explanations can be given for the relatively high signal of the second peak: the claim by the manufacturer that their chemicals are purer than 99% is not true, which is not very likely, or the relative fluorescenc efficiency for the second peak in much higher than that of the first. Because an increase in the ratio of the first to the second peak with a longer fatty acid chain is found, owing to an increase in the second peak, the relative molar fluorescence efficiency of the second peak is not dependent on the mobile phase composition as the BrMMC derivative is[17]. Recrystallization and column chromatography over silica reduced slightly the production of the second peak, but so far no cleaning procedure has given the desired results. Hopefully the structure of the impurity will be elucidated in the near future because it has a high potential as a fluorescence label for acidic ompounds. It possesses better fluorescence than BrMMC, and the relative molar fluorescence is not dependent on the mobile phase composition.

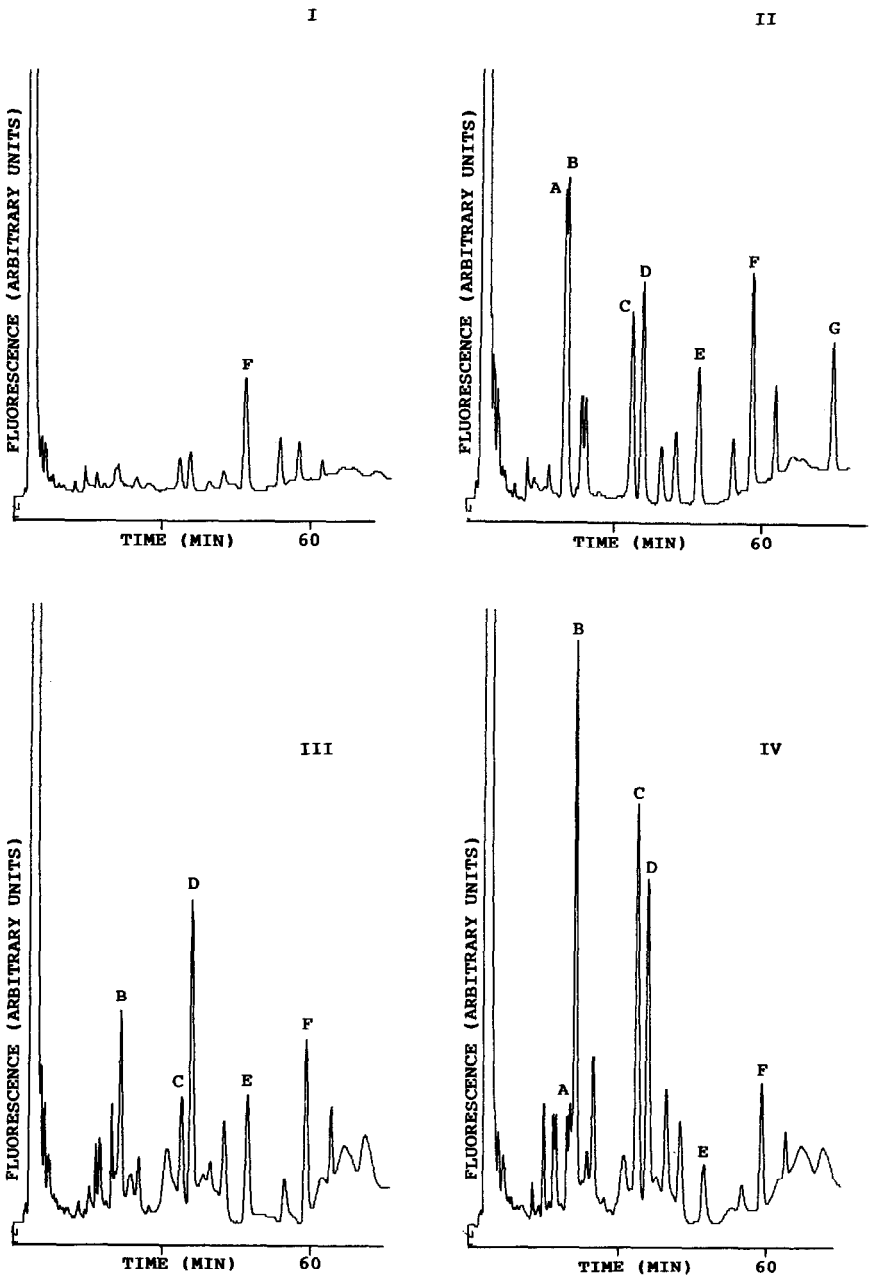

Fig. 3. Chromatograms of derivatized fatty acids taken from a rat during swimming and following rest. (I) Blank; (II) standards; (III) rat after 5 min of swimming; (IV) rat after 20 min of swimming and 10 min of rest. Peaks: A = palmitoleic acid; B = myristic acid; C = oleic acid; D = palmitic acid; E = margaric acid (internal standard); F = stearic acid; G = arachidonic acid. The differences in fatty acid composition and the pharmacological consequences will be discussed in detail elsewhere. The double labelling is not indicated here; for separation conditions, see Experimental.

Application

In Fig. 3 an example of the results of a study of the determination of fatty acids in rat blood in a stress model are shown. In this model rats were initially placed on a platform, which after about 30 min was slowly lowered into a water-bath about 3 m long: the water temperature was 33°C. After lowering of the platform, the rat was forced to swim to the other side of the bath against a water current. After the swim the rat could rest on another platform for 30 min, before being replaced in the home cage. This stress procedure was developed by Scheurink *et al.*[27]. At different time intervals 20 μl of blood were taken for the determination of, among others, fatty acids. The results of these analyses will be published in more detail elsewhere.

CONCLUSION

In bioanalysis, derivatization, either pre- or post-column, is often necessary to achieve better sensitivity; automation could enhance the reproducibility and sample throughput. In a previous paper a general principle for the automated analysis of fatty acids with bromomethylmethoxycoumarin as label was presented[23]. The problems faced are discussed there. In this paper, the optimization of that principle is discussed. By using the Spark PROMIS autosampler, a system could be constructed in which the sample and suspension are placed in the sample vial avoiding contact with tubing other than PTFE, while reducing blanks to a great extent. The application of the procedure has been illustrated by the analysis of rat blood during stress. Previously the usefulness of the automated precolumn derivatization technique for valproic acid has been shown[24].

ACKNOWLEDGEMENTS

This study was supported by the Netherlands Technology Foundation (STW). Spark Holland is thanked for providing the autosampler, Mrs. Veenma-van der Duin for technical assistance and Dr. W. Kuhr for calculations of the reaction kinetics.

REFERENCES

1 N. D. Danielson, M. A. Targove and B. E. Miller, *J. Chromatogr. Sci.*, 26 (1988) 362.
2 N. E. Hoffman, S.-L. Pan and A. M. Rustum, *J. Chromatogr.*, 465 (1989) 189.
3 W. Dünges, *Anal. Chem.*, 49 (1977) 442.
4 W. Voelter, R. Huber and K. Zech, *J. Chromatogr.*, 17 (1981) 491.
5 K. Hayashi, J. Kawase, K. Yoshimura, K. Ara and K. Tsuji, *Anal. Biochem.*, 136 (1984) 314.
6 W. Dünges and N. Seiler, *J. Chromatogr.*, 145 (1978) 483.
7 W. Dünges, *Chromatographia*, 9 (1976) 624.
8 S. G. Zelenski and J. W. Huber, *Chromatographia*, 11 (1978) 645.
9 H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, *J. Chromatogr.*, 234 (1982) 121.
10 E. Grushka, S. Lam and J. Chassin, *Anal. Chem.*, 50 (1978) 1398.
11 K.-E. Karlsson, D. Wiesler, M. Alasandro and M. Novotny, *Anal. Chem.*, 229 (1985) 229.
12 W. Dünges, A. Meyer, K.-E. Müller, M. Müller, R. Pietschmann, C. Plachetta, R. Sehr and H. Tuss, *Fresenius Z. Anal. Chem.*, 288 (1977) 361.
13 E. Bousquet, G. Romeo and L. I. Giannola, *J. Chromatogr.*, 344 (1985) 325.
14 C. Hamada, M. Iwasaki, N. Kuroda and Y. Ohkura, *J. Chromatogr.*, 341 (1985) 426.
15 M. Yamaguchi, S. Hara, R. Matsunaga, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 346 (1985) 227.
16 M. Yamaguchi, R. Matsunaga, S. Hara, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 375 (1986) 27.

17 J. B. F. Lloyd, *J. Chromatogr.*, 178 (1979) 249.
18 W. Elbert, S. Breitenbach, A. Neftel and J. Hahn, *J. Chromatogr.*, 328 (1985) 111.
19 R. Farionotti, Ph. Siard, J. Bourson, S. Kirkiacharian, B. Valeur and G. Mahuzier, *J. Chromatogr.*, 269 (1983) 81.
20 C. Gonnet, M. Marichy and N. Philippe, *Analysis*, 7 (1979) 370.
21 M. Yamaguchi, R. Matsunaga, K. Fukunda and M. Nakamura, *J. Chromatogr.*, 414 (1987) 275.
22 R. A. Kelly. D. S. O'Hara and V. Kelley, *J. Chromatogr.*, 416 (1987) 247.
23 J. H. Wolf and J. Korf, *J. Chromatogr.*, 436 (1988) 437.
24 J. H. Wolf, L. Veenma-van der Duin and J. Korf, *J. Chromatogr.*, 487 (1989) 496.
25 J. H. Wolf and J. Korf, *J. Pharm. Pharmacol.*, in press.
26 S. Lam and E. Grushka, *J. Chromatogr.*, 158 (1978) 207.
27 A. Scheurink, A. B. Steffens, G. H. Dreteler, L. Benthem and R. Bruntink, *Am. J. Physiol.*, 256 (1989) R169.