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USE OF TRIFLUOROACETIC ACID IN THE SEPARATION OF OPIATES AND OPIOID PEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

C. E. DUNLAP III, SUSAN GENTLEMAN and LOUISE I. LOWNEY
Addiction Research Foundation, Palo Alto, Calif. (U.S.A.)
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SUMMARY

Dilute trifluoroacetic acid (TFA) was observed to enhance partition of opioid peptides into butanol during the course of aqueous–organic phase extractions aimed at further purification of pituitary endorphins found in commercial ACTH preparations. Since TFA is volatile and easily removed by lyophilization, it was decided to study TFA as a counter ion for ion-pair partition reversed-phase liquid chromatography.

It was found, that in addition to ion-pair formation, TFA appeared to decrease non-specific adsorption of opiates and opioid peptides to column matrices.

Elution time of opiates and opioid peptides was decreased without loss of resolution when TFA was employed. Peak sharpening and elimination of tailing was observed for several compounds eluted with solvent systems containing TFA.

INTRODUCTION

The development of various types of reversed-phase column packings has extended the application of high-performance liquid chromatography (HPLC) to a number of compounds of biological interest. Reversed-phase liquid chromatographic (LC) separations of opiates and opioid peptides have been reported by several laboratories[1–3].

In general, these compounds exist as cations under the conditions that have most widely been employed in LC. Therefore, partition of these compounds into the non-polar stationary phase may be more effective by suppressing the charge, or by ion-pairing with a suitable counter ion[4]. The alkaline conditions required for ion suppression are generally detrimental to the integrity of silica-gel based packing materials, and thus ion-pairing appears to be the method of choice. Ion-pair partition systems have many desirable properties: high selectivity, stability and, in the presence of a sufficient excess of counter ion, independence of concentration[5]. Commercial ion-pairing reagents are available, but have the major disadvantage that their removal from both the sample and column after chromatography is often extremely difficult.

The effect of dilute trifluoroacetic acid (TFA) in enhancing the partition of

opioid peptides (endorphins) into an organic solvent was observed in this laboratory during efforts to further purify pituitary endorphins present in commercial ACTH preparations[6]. Since TFA is quite volatile and readily removed by lyophilization, it was decided to explore the use of TFA as a counter ion for ion-pair partition reversed-phase LC for separations of opiates and endorphins.

MATERIALS

Solvents for LC included glass-distilled acetonitrile (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.), HPLC grade methanol (Burdick & Jackson Labs.; Fisher Scientific, Fair Lawn, N.J., U.S.A.), 88% reagent grade formic acid (J. T. Baker, Phillipsburg, N.J., U.S.A.) and 99% reagent grade TFA (Aldrich, Milwaukee, Wisc., U.S.A.). Water used to prepare samples and solvents for HPLC was glass-distilled, then treated with activated charcoal (norit A) and filtered through a 0.45-μm Millipore mixed cellulose acetate and nitrate membrane filter. All aqueous solvents were also refiltered just prior to use.

Spectrophotometric grade *n*-butanol was obtained from J. T. Baker, Acthar (corticotropin for injection) 40 U.S.P. units per vial from Armour Pharmaceutical (Phoenix, Ariz., U.S.A.) and synthetic camel β-endorphin from Pierce (Rockford, Ill., U.S.A.).

2,9-β-Dimethyl-2'-hydroxy-6,7-benzomorphan HBr (DMHBM) and 2,9-β-dimethyl-6,7-benzomorphan HCl (DMBM) were gifts from the National Institutes of Health. N-Allylnormetazocine (SKF 10047), ketocyclozocine (KC), and ethylketocyclazocine (EKC) were gifts from Dr. W. Martin, Addiction Research Center (Lexington, Ky., U.S.A.). A solution of the five benzomorphans was made up in water to final concentrations $4 \cdot 10^{-4}$ *M* DMHBM, $6 \cdot 10^{-4}$ *M* DMBM, $8.6 \cdot 10^{-5}$ *M* SKF 10047 and $2 \cdot 10^{-4}$ *M* KC and EKC. l-Methadone HCl was obtained from Eli Lilly (Indianapolis, Ind., U.S.A.) and made up to a 10 m*M* aqueous solution.

METHODS

HPLC was performed with a Waters Assoc. system consisting of two Model 6000A solvent delivery pumps, a U6K universal injector, a Model 660 solvent programmer and a Model 440 absorbance detector at 254 nm. Either a 4 mm $\times$ 30 cm C_{18} or alkylphenyl reversed-phase μBondapak column (Waters Assoc., Milford, Mass., U.S.A.) was used, as noted in specific experiments. The aqueous phase of the LC solvent system was pumped through one pump and the organic phase through the second pump, with the final solvent composition controlled by the solvent programmer.

Opioid activity was assayed using the guinea pig ileum–myenteric plexus preparation[7].

EXPERIMENTAL

One vial of Armour Acthar (40 U.S.P. units of ACTH containing 14 mg hydrolyzed gelatin) was dissolved in 0.5 ml of glass-distilled water. Aliquots of 25 μl of this solution were added to 1 ml each of 0.1 *M* acetic acid, 0.1 *M* formic acid,

0.1 *M* tris–HCl (pH 7.4), or 1% TFA. All aqueous phases had been previously equilibrated with *n*-butanol. A 1-ml volume of *n*-butanol was then added to each of the aqueous phases and all phases mixed by vortexing for 1 min.

After separation by low speed centrifugation, 0.5 ml of each phase was lyophilized, then taken up in a small volume of 0.01 *M* HCl and assayed for opioid activity in the guinea pig ileum preparation. Percent recoveries were determined by comparison with dose response curves obtained for each ileum strip using the original Armour ACTH preparation, which contains endorphin as a contaminant.

Samples of synthetic β-endorphin were chromatographed on a Waters Assoc. reversed-phase μBondapak C_{18} column. A sample of 2.5 nmoles of β-endorphin was eluted with 5 m*M* TFA, pH 2.5, using a linear gradient from 30 to 50% acetonitrile, with all effluent fractions being collected. The column was then washed with 10 to 15 column volumes of 30% acetonitrile in 10 m*M* formic acid, pH 2.8. A second 2.5-nmole sample was applied and eluted using a linear gradient from 30 to 70% acetonitrile in 10 m*M* formic acid, and the effluent fractions collected. All fractions collected were assayed on the guinea pig ileum preparation to determine recovery of opioid activity.

LC separation of the mixture of five benzomorphans was carried out using a Waters Assoc. reversed-phase alkylphenyl μBondapak column. A 10-μl volume of the benzomorphan solution was eluted isocratically using a 50% methanol in 10 m*M* formic acid solvent system at a flow-rate of 1 ml/min. The column was then re-equilibrated with 50% methanol in 5 m*M* TFA, and a second 10-μl sample of the benzomorphan solution was injected and eluted isocratically.

This experimental procedure was repeated for a 4-μl sample of 10 m*M* l-methadone HCl, except that the methanol concentration was increased to 60% in either 10 m*M* formic acid or 5 m*M* TFA.

RESULTS AND DISCUSSION

The opioid peptides present in commercial ACTH preparations, having apparent molecular weights from 1700 to 3300 daltons, partition very poorly into *n*-butanol from acidic or neutral solutions (Table I). Addition of 1% TFA resulted in 50% recovery of the opioid activity in the *n*-butanol phase after one extraction, and 90% of the total activity was recovered in butanol after three extractions. Some

TABLE I

PARTITION OF PITUITARY ENDORPHINS INTO *n*-BUTANOL FROM VARIOUS AQUEOUS PHASES

Aqueous phase	*Initial activity in aqueous phase (%)* *	*Initial activity in butanol phase (%)* *
0.1 *M* Acetic acid	96	0
0.1 *M* Formic acid	90	0
0.1 *M* tris–HCl pH 7.4	90	0
1% TFA	43	50

* Percent recoveries were determined by guinea pig ileum bioassay as reported in the text. Activity recovered from each phase was compared with dose response curves determined for each ileum strip from the original material.

partitioning into butanol occurred under more alkaline conditions (1% NH_4OH–pyridine–butanol, 11:3:5), but opioid activity was found to be unstable in this pH range, and recovery was poor. The results obtained from aqueous phase–butanol extractions indicate that the enhanced lipophilicity of endorphins in the presence of TFA is the result of ion-pair partition, and not simply due to the acidic conditions, since no partitioning is seen to occur from 100 m*M* acetic or formic acid.

LC elution patterns of β-endorphin from a reversed-phase C_{18} column are shown in Fig. 1. A symmetrical peak of UV-absorbing material eluting at 6 min during the acetonitrile–5 m*M* TFA gradient was associated with opioid activity (Fig. 1a). Recoveries of applied opioid activity as demonstrated by guinea pig ileum bioassay were 79 and 100% in two experiments. No UV-absorbing peak or opioid activity was observed when β-endorphin was eluted with an acetonitrile–10 m*M* formic acid

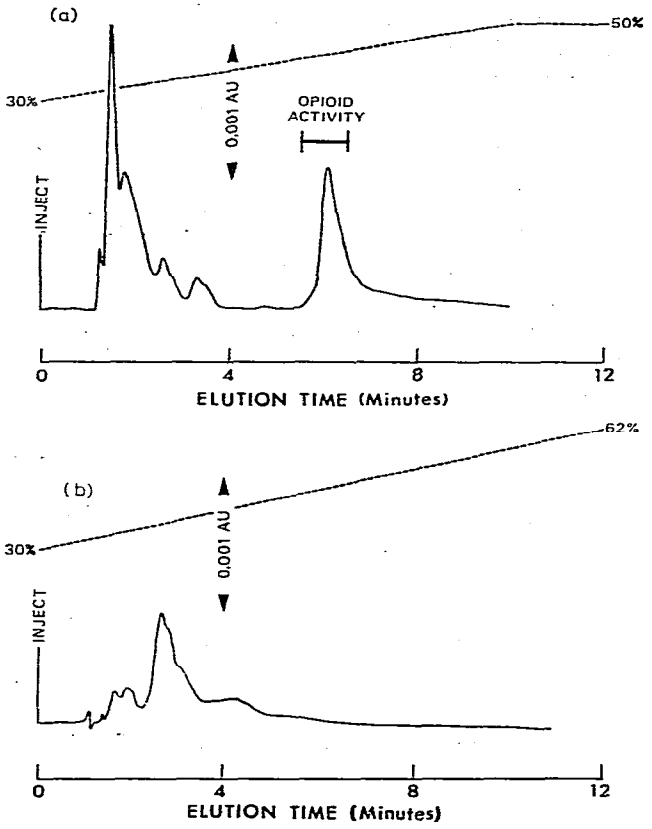

Fig. 1. (a) Elution of β-endorphin with acetonitrile–TFA. A 25-μl, volume of 10^{-4} *M* β-endorphin was applied to a reversed-phase C_{18} column and eluted with a 10-min linear gradient from 30 to 50% acetonitrile in 5 m*M* TFA at 25° with a flow-rate of 2 ml/min (approximately 1200 p.s.i.). Absorbance due to the solvent blank has been subtracted from the chromatogram, and the gradient is indicated by a dashed line. (b) Elution of β-endorphin with acetonitrile–formic acid. A 25-μl volume of 10^{-4} *M* β-endorphin was applied to a reversed-phase C_{18} column and eluted with a 15-min linear gradient from 30 to 70% acetonitrile in 10 m*M* formic acid at 25° with a flow-rate of 2 ml/min (approximately 1200 p.s.i.). Absorbance due to the solvent blank has been subtracted from the chromatogram, and the gradient is indicated by a dashed line.

gradient (Fig. 1b). However, a 2-ml wash of 70% acetonitrile in 4 m*M* TFA injected at the end of the acetonitrile–formic acid gradient eluted 73% of the applied opioid activity (data not shown).

If 10-fold larger amounts of β-endorphin are injected, some opioid activity (30–60%) is eluted at 6 min with the acetonitrile–formic acid gradient, but the activity peak tends to trail badly (data not shown).

The data suggest that loss of β-endorphin on the C_{18} column is due to non-specific adsorption to sites on the silica gel. The interaction between β-endorphin and these column adsorption sites is eliminated in the presence of TFA, resulting in improved resolution with sharper and more symmetrical peaks. In general, the addition of an ion-pairing reagent will increase the retention times of samples with which it pairs in an HPLC system in which all other parameters remain constant[8]. However, the retention of β-endorphin on a reversed-phase C_{18} column is greatly decreased when eluted with an acetonitrile–TFA solvent system instead of an acetonitrile–formic acid solvent system. This is probably due to decrease in adsorption of β-endorphin to the column matrix following ion-pairing with TFA.

The success of TFA in improving the resolution of complex mixtures of basic peptides separated by HPLC suggested its use in HPLC separation of opiate drugs, which also contain a positive charge at pH 7 or less. Separations of a solution containing a mixture of five benzomorphans were carried out isocratically in a solvent system of 50% methanol in 10 m*M* formic acid and in 50% methanol in 5 m*M* TFA (Fig. 2).

The capacity factor, k', of a peak eluted by HPLC is defined as

$$k' = \frac{V_e - V_0}{V_0} \tag{1}$$

where V_e is the elution volume of the eluted peak, and V_0 is the void volume of the column (the volume in which a peak would elute if it were completely unretained by the column). The selectivity of separation factor, α, is the relative retention of one substance compared to the substance eluting just before it, and can be expressed as

$$\alpha = \frac{V_{e_2} - V_0}{V_{e_1} - V_0} \text{ or } \frac{k'_{e_2}}{k'_{e_1}} \tag{2}$$

When eluted with 50% methanol in 10 m*M* formic acid, all five drugs were extremely well resolved. With this system the alkylphenyl column displayed a high capacity for the five solutes, with k' values ranging from 3.78 to 12.75 (Table II). When 50% methanol in 5 m*M* TFA is substituted for methanol in formic acid, the selectivity or relative retention for each pair of peaks remains about the same, but the capacity of the column is greatly reduced (Table II). Capacity factors range from 1.17 to 4.17 with the methanol TFA solvent system (Table II). The most striking difference between the two solvent systems was observed in the efficiency of the alkylphenyl column. The number of theoretical plates, N, which is a measure of column efficiency, is practically defined as

$$N = 16\frac{V_e^2}{W_e} \tag{3}$$

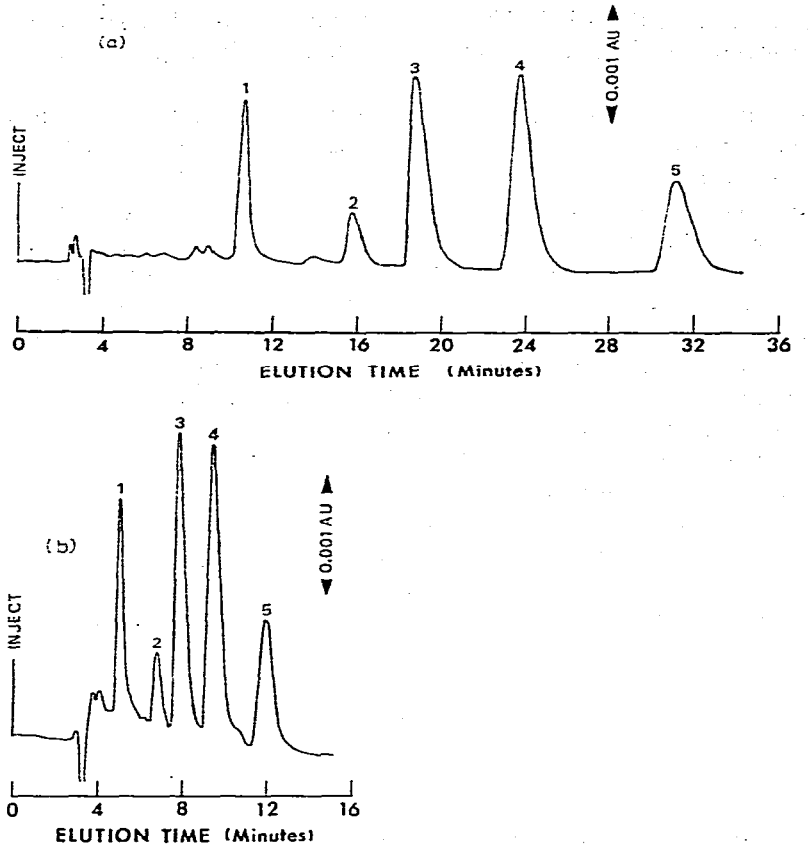

Fig. 2. (a) Separation of 5-benzomorphan mixture using methanol–formic acid. A 10-μl volume of a sample containing $4 \cdot 10^{-4}$ *M* dimethylhydroxybenzomorphan (1), $8.6 \cdot 10^{-5}$ *M* N-allylnormetazocine (2), $6 \cdot 10^{-4}$ *M* dimethylbenzomorphan (3), $2 \cdot 10^{-4}$ *M* ketocyclazocine (4) and ethylketocyclazocine (5) was eluted isocratically from a reversed-phase alkylphenyl column with 50% methanol in 10 m*M* formic acid at 25° with a flow-rate of 1 ml/min (approximately 500 p.s.i.). (b) Separation of a 5-benzomorphan mixture using methanol–TFA. A 10-μl volume of the same 5-benzomorphan mixture used in (a) was eluted isocratically from an reversed-phase alkylphenyl column with 50% methanol in 5 m*M* TFA at 25° with a flow-rate of 1 ml/min (approximately 500 p.s.i.). 1 = Dimethylhydroxybenzomorphan, 2 = N-allylnormetazocine, 3 = dimethylbenzomorphan, 4 = ketocyclazocine and 5 = ethylketocyclazocine.

where V_e, as previously defined, is the elution volume of a test sample and W_e is the width of the peak, in volume units, defined as the distance between the points at which tangents drawn to the inflection points of the two sides of the peak, intersect the baseline. The height equivalent to a theoretical plate, HETP, is simply the column length in millimeters divided by N. The number of theoretical plates for the alkylphenyl column for each solvent system was arbitrarily calculated from measurements of the first peak eluted. The number of theoretical plates calculated for the methanol–formic acid solvent system was 2415 (HETP = 0.12 mm). When methanol–TFA is used, this value was reduced by half to 1220 (HETP = 0.24 mm).

The separation factors for each pair of drugs did not change when formic acid

TABLE II

CALCULATED k' AND α VALUES FOR SEPARATION OF BENZOMORPHANS USING BOTH 50% METHANOL IN 10 m*M* FORMIC ACID AND 50% METHANOL IN 5 m*M* TFA SOLVENT SYSTEMS*

Drug	k' *Methanol in HCOOH*	k' *Methanol in TFA*	α *Methanol in HCOOH*	α *Methanol in TFA*
Dimethylhydroxybenzomorphan	3.78	1.17		
			1.59	1.67
N-Allylnormetazocine	6.00	1.94		
			1.21	1.23
Dimethylbenzomorphan	7.28	2.38		
			1.30	1.29
Ketocyclazocine	9.47	3.08		
			1.35	1.35
Ethylketocyclazocine	12.75	4.17		

* The terms k' and α are defined under Results and discussion.

was replaced by TFA. This observation together with the decrease in both efficiency and capacity of the alkylphenyl column, indicates that TFA acts by some mechanism to decrease interactions of the solutes with the stationary phase of the column. For this mixture of benzomorphans, 50% methanol in 10 m*M* formic acid actually provides better separation of the drug mixture.

A slightly different case is seen when the two solvent systems are compared for the elution of l-methadone HCl. Elution of methadone with 60% methanol in 10 m*M* formic acid produces an unsymmetrical peak with a large amount of tailing (Fig. 3a). The capacity factor is 9.17, resulting from a fairly long retention. The calculated

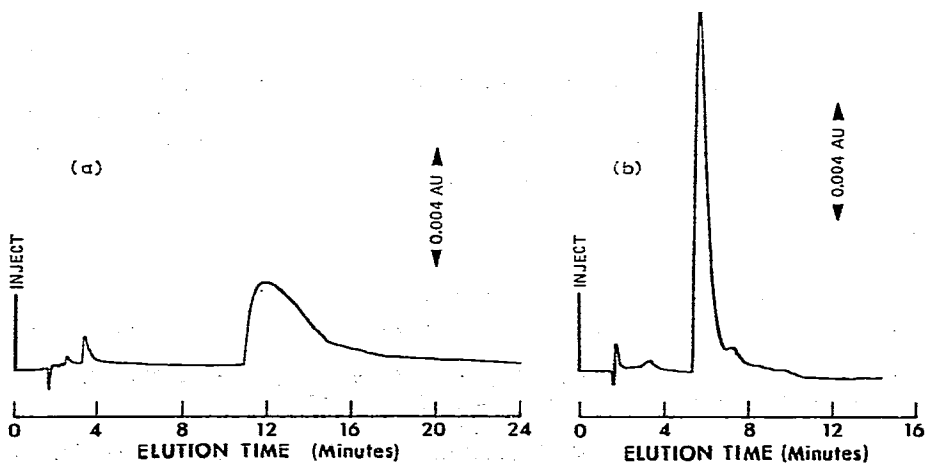

Fig. 3. (a) Elution of 1-methadone with methanol–formic acid. A 4-μl volume of 10 m*M* 1-methadone HCl was eluted isocratically from a reversed-phase alkylphenyl column with 60% methanol in 10 m*M* formic acid at 25° with a flow-rate of 2 ml/min (approximately 1000 p.s.i.). (b) Elution of 1-methadone with methanol–TFA. A 4-μl volume of 10 m*M* 1-methadone HCl was eluted isocratically from a reversed-phase alkylphenyl column with 60% methanol in 5 m*M* TFA at 25° with a flow-rate of 2 ml/min (approximately 1000 p.s.i.).

number of theoretical plates for this system is only 88 (HETP = 3.41 mm) due to the extreme peak broadening. When formic acid is replaced with 5 m*M* TFA, a dramatic sharpening of the methadone peak is obtained (Fig. 3b). As with the benzomorphans, the retention is greatly decreased, producing a capacity factor of 3.83. However, the calculated number of theoretical plates is increased by almost 5-fold to 410 (HETP = 0.73 mm). Here again, TFA appears to decrease interaction between methadone and non-specific adsorptive sites on the matrix of the stationary phase. The increase in column efficiency in the presence of TFA results from peak sharpening and elimination of tailing.

CONCLUSIONS

TFA was observed to act as an ion-pairing agent in the butanol extraction of pituitary endorphins, which led to its study as a potentially useful counter ion for non-destructive ion-pair partition reversed-phase LC, since it can readily be removed from chromatographed samples by evaporation or lyophilization. The work presented here indicates that ion-pair formation of opiates and opioid peptides with TFA produces a decrease in their interactions with reversed-phase C_{18} or alkylphenyl columns, and this is postulated to be due primarily to elimination of adsorption of the compounds to column matrices.

Therefore, TFA is a potentially useful reagent for HPLC, in that it decreases elution time without any loss of resolution, as opposed to increasing organic solvent concentration to decrease elution time. It may also improve sensitivity and resolution for many compounds which are adsorbed to silica, through peak sharpening and elimination of tailing.

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