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USE OF LIQUID CHROMATOGRAPHY IN THE SYNTHESIS OF ISOLUMINOL-LABELLED MEDROXYPROGESTERONE ACETATE AND ZERANOL

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for monitoring the syntheses of two isoluminol-labelled drugs, medroxyprogesterone acetate (MPA) and zeranol, has been developed. MPA and the ketone derivative of zeranol, zearalanone, were conjugated to N-(4-aminobutyl)-N-ethylisoluminol through the carboxymethyloxime derivative of the drug by using the N-succinimide ester as an intermediary. Reaction mixtures were sampled periodically and chromatographed directly by HPLC on a silica gel column, by using isocratic elution with mixtures of hexane–ethanol–acetic acid in several different proportions. The degree of reaction completion was determined by comparison of the peak area of the initial reactant to that present at sampling time. MPA oxime production was found to be complete after 15 min; 97.0% of the oxime was converted to succinimide ester in 24 h; 99.0% of the available ester reacted within 2.5 h to form the final labelled product. Zearalanone oxime production was found to be complete after 2 h; 93.3% of the oxime was converted to the activated ester within 24 h; 89.6% of available ester had reacted in 30 min to form the final labelled product. The chromatography can be performed in real time, permitting modification in the conditions of the reaction while in progress.

INTRODUCTION

Luminescence immunoassay (LIA), a novel technique in which the labelled ligand bears a chemiluminescent group, is gaining popularity. One of the reasons for its increasing popularity is that, although the labelled ligand, and sometimes even the labelling reagent, are often unavailable commercially, they can be synthesized with relative ease in an average chemical laboratory.

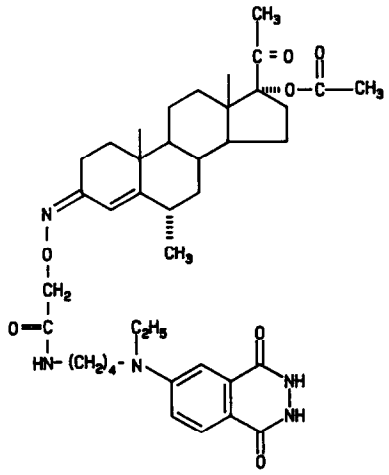
Quantitation and identification of synthesized products have commonly been achieved with purification by recrystallization and weighing, followed by mass spectrometry (MS) or nuclear magnetic resonance (NMR)[1–3]. The required purification procedures, although well established, are often lengthy and difficult to perform. Such a method is therefore not suitable for following the reaction progress.

Traditionally, thin-layer chromatography has been used to monitor reactions[4] with acceptable results. However, this procedure suffers from several disadvantages:

(1) the results are only qualitative; (2) partially reacted material may be driven to completion during spotting of the plates[5], thus giving false impressions of the reaction kinetics; (3) plates contaminated by normal laboratory atmosphere may obscure the results[6].

High-performance liquid chromatography (HPLC) has been effectively utilized in the quality control and purification of luminogenic labels[7,8]. This prompted us to examine HPLC for monitoring the progress of reactions involved in the synthesis of chemiluminescent labels. Immediate information on purity, yield, and identification of sample components is obtained through analysis of the crude reaction mixture at the start and fisnish of each intermediate step. In this way, the necessity for tedious purification procedures can be eliminated.

In this paper, the HPLC technique developed for monitoring the isoluminol derivatization of two drugs, zeranol (Ralgro) and medroxyprogesterone acetate (MPA) (Provera), is presented (Fig. 1).



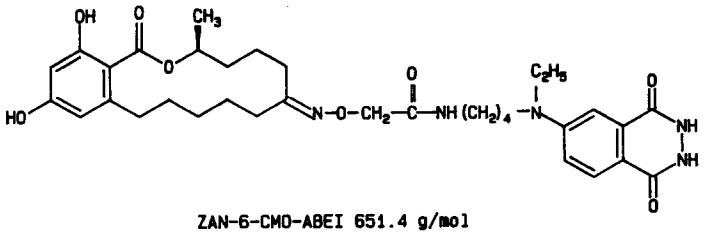


Fig. 1. Structural formulae of isoluminol-labelled medroxyprogesterone acetate (MPA-3-CMO-ABEI) and zeranol (ZAN-6-CMO-ABEI). Zearalanone (the ketone form of zeranol) was used to form the carboxymethyloxime derivative.

EXPERIMENTAL

Chemicals

Zeranol and zearalanone (ZAN) were kindly provided by the International Minerals and Chemical Corporation (Terre Haute, IN, U.S.A.), and MPA by Upjon (Kalamazoo, MI, U.S.A.). Carboxymethoxylamine hemihydrochloride, N-hydroxysuccinimide and 1,3-dicyclohexylcarbodiimide were purchased from Aldrich (Milwaukee, WI, U.S.A.), and N-(4-aminobutyl)-N-ethylisoluminol (ABEI) from Sigma (St. Louis, MO, U.S.A.). Other reagents of analytical grade were obtained from various commercial sources.

Apparatus

The following apparatus were used: HPLC, a Waters Assoc. liquid chromatography system consisting of a Model 6000A solvent delivery system, Model U6K injector, and Model 440 ultraviolet detector; NMR, Bruker AM-400, measuring protons at 400.13 MHz; MS, Finnigan MAT, Model 4610, used in the chemical ionization (CI) mode, with solid probe at 260°C.

Preparation of MPA-3-carboxymethyloxime-ABEI

The procedure used was a modification of the ones described by Jansen *et al.*[8,9] and Cornette *et al.*[10]. A solution of MPA (0.5 mmol) in pyridine (15.0 ml) was reacted with carboxymethoxylamine (CMO) hemihydrochloride (450 mg, equivalent to 4 mmol CMO), with stirring for 15 min at room temperature. The pyridine was removed in a rotary evaporator under reduced pressure. The residue was taken up in cold 0.1 *M* HCl (30 ml), and the acidified oxime was extracted in ethyl acetate (3 × 40 ml). The combined extracts were washed with water (3 × 15 ml), and dried with anhydrous sodium sulphate. The solvent was evaporated as above, leaving MPA-3-CMO as a white foam (250 mg yield).

MPA-3-CMO (68.9 mg, 0.15 mmol) was dissolved in anhydrous dimethylformamide (1.5 ml). Anhydrous N-hydroxysuccinimide (17.3 mg, 0.15 mmol) was added and stirred to dissolve. The solution was cooled to 0°C and 1,3-dicyclocarbodiimide (30.95 mg) was added. After 24 h at room temperature, the activated ester supernatant was syphoned off with a Pasteur pipette from the crystalline dicyclohexylurea by-product.

To the activated ester solution was added dropwise 1 equivalent of ABEI dissolved in 0.2 *M* sodium dihydrogenphosphate (3.0 ml, final pH 7.5). Ethyl acetate (6.0 ml) was added and the resulting clear two-phase reaction mixture was stirred for 2.5 h. The organic layer was collected, and the aqueous phase was washed with ethyl acetate (2 × 3.0 ml). The organic extracts were combined, dried by adding anhydrous sodium sulphate, filtered and evaporated leaving MPA-CMO-ABEI (81 mg) as a yellow syrup.

Preparation of ZAN-6-CMO-ABEI

The isoluminol-labelled zeranol was prepared by the method of Jansen *et al.*[9] using ZAN as starting material and four-fold excess equivalents of CMO hemihydrochloride. The final product was extracted in ethyl acetate (1.0 ml), dehydrated by mixing with sodium sulphate, filtered and evaporated to *ca.* 0.2 ml, yielding fine pale yellow crystals at 4°C.

Chromatographic procedure

Normal-phase HPLC was performed with a column (15 cm × 4.6 mm I.D.) of silica gel, particle size 5 μm (S5W, Chromatography Sciences, Montreal, Canada). Four different combinations of hexane–ethanol–acetic acid were used under the following conditions: 92:8:0.5 (v/v/v) at 2.0 ml/min for monitoring the reaction kinetics of MPA, MPA-3-CMO, and MPA-3-CMO-activated ester; 75:25:0.5 (v/v/v) at 2.0 ml/min for MPA-3-CMO-ABEI; 95:5:0.5 (v/v/v) at 2.0 ml/min for ZAN and ZAN-6-CMO; and 80:20:0.5 (v/v/v) at 3.0 ml/min for ZAN-6-CMO-activated ester and ZAN-6-CMO-ABEI. Column eluent was monitored at 254 nm for MPA and its derivatives, and at 313 nm for ZAN and its derivatives.

At each stage of preparation, samples (1 μl) of the reaction mixture were removed for HPLC analysis starting at time 0 h and continuing periodically until the reaction reached a steady state. The percent completion of each reaction was estimated from the peak area ratios of the starting material at the beginning and the end of the reaction. Overall yields were calculated by using the expected and actual weights of the finished products.

For both drugs the starting material, the oxime and the finished product were identified by MS and NMR. The identity of the activated ester peak on HPLC was assumed from its rate of formation, with the simultaneous disappearance of the starting materials.

RESULTS

Reaction progress was monitored by HPLC as described in the Experimental section. For each drug, at least two mobile phases were required for adequate separation of all derivatives (Table I). The oxime of both drugs, and the ester of MPA, were found to elute as double peaks representing *syn*- and *anti*-isomers at the oxime nitrogen. The presence of the two isomers was confirmed by NMR. Two peaks close together, near 4.5 ppm, were present at a ratio of approximately 2:1 in both

TABLE I

CAPACITY FACTORS (k') OF MEDROXYPROGESTERONE ACETATE, ZEARALANONE AND THEIR DERIVATIVES

Column: silica gel, 5 μm, 150 × 4.6 mm I.D. Mobile phase A: hexane–ethanol–acetic acid (92:8:0.5, v/v/v), 2.0 ml/min; mobile phase B: hexane–ethanol–acetic acid (75:25:0.5, v/v/v), 2.0 ml/min; mobile phase C: hexane–ethanol–acetic acid (95:5:0.5, v/v/v), 2.0 ml/min; mobile phase D: hexane–ethanol–acetic acid (80:20:0.5, v/v/v), 3.0 ml/min.

Derivative	*MPA*		*ZAN*	
	A	*B*	*C*	*D*
Drug	3.0	–	1.8	–
Oxime	2.1, 2.6[a]	1.0	2.4, 2.9[a]	1.3
Succinimide ester	6.3, 7.6[a]	1.4	12.0	1.8
Final product				
Drug-CMO-ABEI	–	2.7	–	4.3

[a] Two isomers (*syn* and *anti*).

MPA-3-CMO-ABEI and ZAN-6-CMO-ABEI, representing the methylene protons of the CMO residue.

Oxime formation

The oximes of MPA and ZAN were prepared by reacting each drug with a 4–8 fold excess carboxymethoxylamine in pyridine at room temperature. Excess pyridine had a tendency to interfere with chromatography, but this could be controlled by diluting the reaction mixture 1:400 in hexane and injecting 20 μl. Based on comparison of initial reactant to residual amounts, MPA-3-CMO production was found to be complete within 15 min in presence of 4 mmol CMO (Fig. 2A and B). MPA was well separated from MPA-3-CMO, forming a shoulder on the descending side of the oxime doublet (Fig. 2C). The limit of detection of residual MPA from amongst the oxime trailing peak was estimated to be approximately 5% of the total oximes.

The formation of ZAN-6-CMO was also rapid at a CMO:ZAN ratio of 4 (Fig. 3). Over 70% of ZAN had reacted in 15 min, and the reaction was essentially 100% complete after 2 h at room temperature.

The oxime was purified by evaporating the reaction mixture, partitioning the residue between ethyl acetate and an excess of cold 0.1 *M* HCl and evaporating the extract. In these conditions, no hydrolysis of the oxime was observed as seen by the

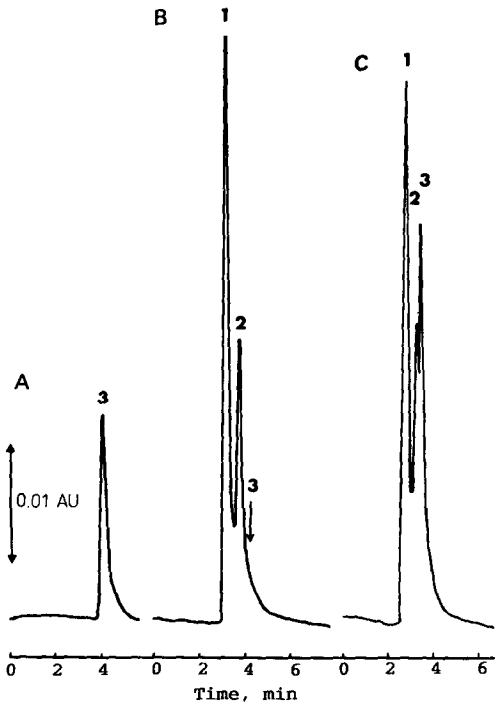


Fig. 2. Reaction of MPA with carboxymethoxylamine. Reaction times: (A) 0 h; (B) 0.25 h; (C) MPA and MPA-3-CMO. Peaks: (1) and (2), MPA-3-CMO (*syn* and *anti* isomers), (3) MPA. Chromatographic conditions: column, silica gel (5 μm particles, 150 × 4.6 mm I.D.). Mobile phase, hexane–ethanol–acetic acid (92:8:0.5, v/v/v), at 2.0 ml/min. Detection at 254 nm.

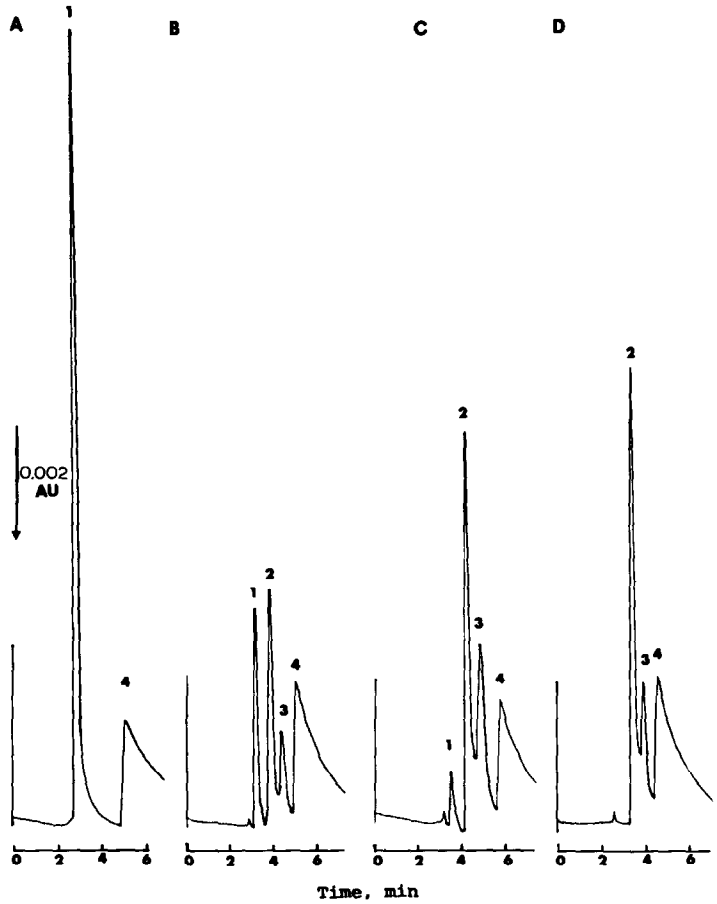


Fig. 3. Reaction of zearalanone with carboxymethoxylamine. Reaction times: (A) 0 h; (B) 0.25 h; (C) 1 h; (D) 2 h. Peaks: (1) ZAN; (2) and (3) ZAN-6-CMO (*syn* and *anti* isomers); (4) pyridine. Mobile phase, hexane–ethanol–acetic acid (95:5:0.5, v/v/v). Detection at 313 nm. Other conditions as in Fig. 2.

absence of either zeranol or ZAN on chromatographing the extract. Based on final weights, recovery of oxime product was 100% for MPA-3-CMO, and 87.1% for ZAN-6-CMO (Table II).

Formation of the succinimide ester

This reaction was accomplished using N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide. Upon reaction, both oxime peaks disappeared and were replaced by the succinimide ester peaks as illustrated in Fig. 4 for MPA. For both drugs, a steady state was reached within 24 h. In this time period, based on peak areas, 97.0% of the MPA-3-CMO and 93.3% of ZAN-6-CMO had reacted.

Formation of the labelled product

The succinimide ester derivative of each drug was reacted with a slight excess of ABEI in a two-phase system consisting of dibasic phosphate and ethyl acetate. Both

TABLE II

RECOVERY OF MPA AND ZERANOL DERIVATIVES

Compound	*MW*	*Weight (mg)*	*mmol*	*% yield*
Zearalanone	320.4	160.1[a]	0.4997	–
ZAN-6-CMO	393.6	171.2[b]	0.4350	87.1
ZAN-6-CMO	393.6	50.0[a]	0.1270	–
ZAN-6-CMO-ABEI	651.9	50.4[b]	0.0773	60.9
MPA	386.5	192.7[a]	0.4986	–
MPA-3-CMO	459.7	246.0[b]	0.5351	107.3
MPA-3-CMO	459.7	72.0[a]	0.1566	–
MPA-3-CMO-ABEI	718.0	81.0[c]	0.1128	72.0

[a] Starting material.
[b] By extraction and recrystallization.
[c] By preparative HPLC and evaporation.

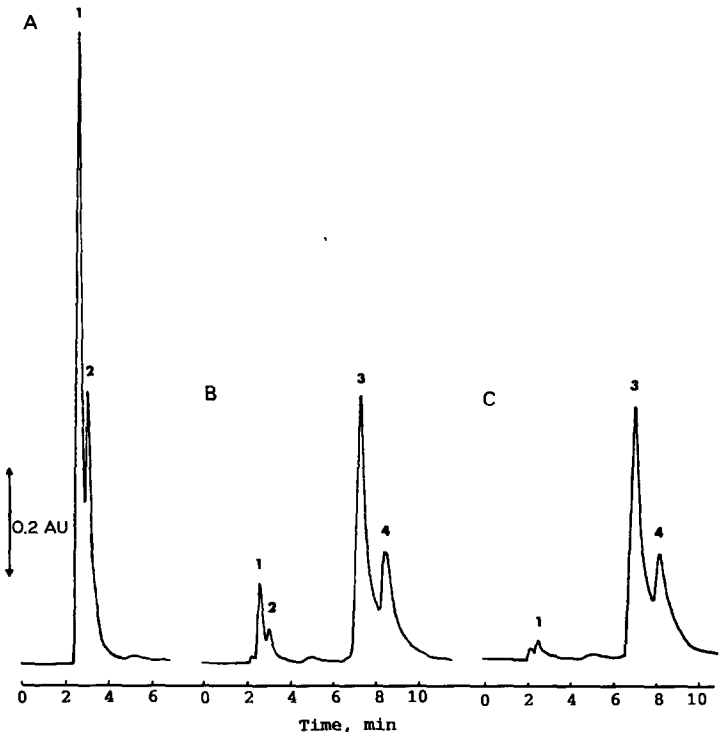


Fig. 4. Reaction of MPA-3-CMO with N-hydroxysuccinimide. Reaction times: (A) 0 h; (B) 6 h; (C) 23 h. Peaks: (1) and (2) MPA-3-CMO (*syn* and *anti* isomers); (3) and (4) MPA-3-CMO-N-succinimide ester (*syn* and *anti* isomers). Chromatographic conditions as in Fig. 2.

succinimide ester peaks reacted to form a single peak of labelled drug as shown in Fig. 5 for MPA. No attempts were made to separate the two isomers. Labelling of MPA was found to reach steady state within 2.5 h with 99.0% of available MPA-3-CMO-N-succinimide ester reacted. Extraction with ethyl acetate afforded 78.4% recovery of the total labelled MPA present in the reaction mixture, as monitored by HPLC. The identity of the product was confirmed by MS in the chemical ionization (CI) mode, showing the [M + 1] peak at *m/z* 718.8, and by NMR, showing the aromatic protons of isoluminol (two doublets at 7 and 8 ppm and a singlet at 7.2 ppm), the CMO methylene protons at 4.5 ppm (*syn* and *anti*, see above) and the aliphatic and steroid protons between 0 and 2.2 ppm.

ZAN-6-CMO-succinimide ester was reacted with ABEI for only 30 min. HPLC analysis of the reaction mixture at this time revealed 89.6% reaction of the available ZAN ester. The efficiency of the extraction of the total labelled ZAN was 72.8%, similar to that of MPA. The final labelled product was fairly pure, with only two small contaminating peaks observable by HPLC analysis.

Fig. 6 illustrates the rates of formation of MPA-3-CMO, MPA-3-CMO-N-succinimide ester and MPA-3-CMO-ABEI from their respective precursors, based on HPLC measurement of the starting material (drug, oxime or activated ester) remaining at different sampling times. The oxime production (top curve) was rapid; steady state was achieved within the shortest sampling period. Production of esterified oxime (bottom curve) was much more gradual taking 23 h for the complete reaction.

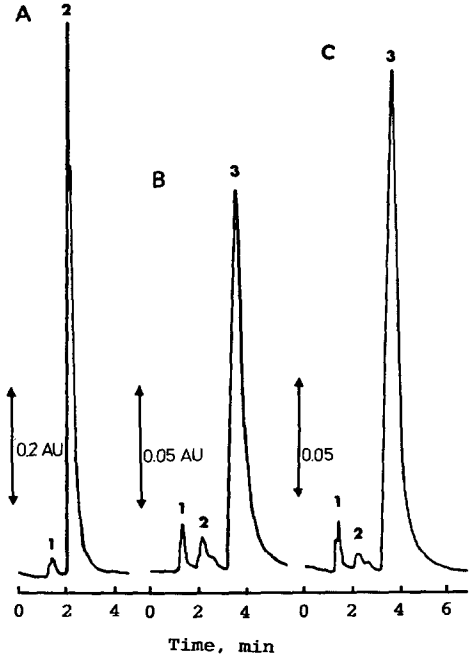


Fig. 5. Reaction of MPA-3-CMO-N-succinimide with ABEI. Reaction times: (A) 0 h; (B) 1.5 h; (C) 2.5 h. Peaks: (1) MPA-3-CMO; (2) MPA-3-CMO-N-succinimide ester (*syn* and *anti* isomers); (3) MPA-3-CMO-ABEI. Mobile phase, hexane–ethanol–acetic acid (75:25:0.5, v/v/v). Other conditions as in Fig. 2.

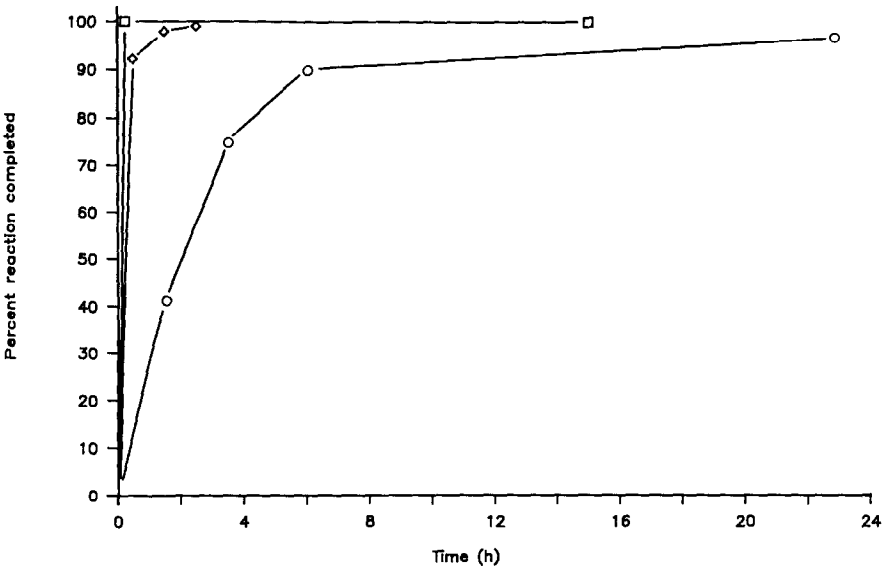


Fig. 6. Reaction profile of the formation of medroxyprogesterone acetate derivatives, estimated from the disappearance of the starting material. (□) MPA-3-CMO; (◇) MPA-3-CMO-ABEI; (○) MPA-3-CMO-N-succinimide ester.

Conjugation to ABEI (middle curve) was intermediate taking 2.5 h for completion. Eventually, each reaction achieved close to 100% yield.

Based on final weights of the products, the yields of both labelled drugs from the oxime were acceptably high: 72.0% for MPA and 60.9% for ZAN (Table II).

DISCUSSION

Monitoring reaction progress by HPLC is a novel technique. HPLC has traditionally proved useful in the isolation, quantitation, and identification of compounds. Here, it was shown to be a viable alternative to conventional monitoring procedures such as thin-layer chromatography. With real-time monitoring by HPLC, quantitative information on product quality and yield is obtained more rapidly, optimum time required for each reaction may be established and low yield can immediately be traced to its source. Thus, the procedure may be quickly modified and improved.

Normal phase chromatography was preferred in most of this work, as this gave good separations, and required only a small number of different mobile phases for all the reactants and products involved. Additionally, the organic mobile phase was compatible with the reaction and extraction (mostly ethyl acetate) for direct injection of the aliquots. Nevertheless, for the final purification of the labelled drug before LIA, a reversed-phase system was found preferable.

The isocratic HPLC system utilized here, although simple, was effective in resolving each of the compounds synthesized. Two mobile phases were, however, required for each drug. Improved monitoring might be achieved with gradient-elution HPLC such that all derivatives of a drug would be analyzed in a single system.

HPLC was effective in monitoring the oxime production of both drugs. The two isomers, *syn* and *anti*, were well separated. Thus, preparative HPLC could be used for separating them, should this be necessary. Separation between the MPA and its oximes was not baseline but still adequate to detect approximately 5% residual drug. With this technique it was found that the time needed for complete synthesis was only 15 min for MPA-3-CMO, and 2 h for ZAN-6-CMO. Others have found that progesterones and ZAN required much longer incubation times, ranging between 16 and 48 h[8–10].

Recovery of MPA-3-CMO was slightly better than that of ZAN-6-CMO. This may have been due to the use of ethyl acetate for extracting the former as opposed to chloroform used for the latter oxime. Both oximes were found to be more soluble in ethyl acetate than in either chloroform or ether, solvents recommended by other authors[6,7].

The conjugation of the activated MPA to ABEI required 2.5 h for completion, although at 30 min it was over 90% complete. The analogous reaction with ZAN, terminated at 30 min, twice the length of time suggested by Jansen *et al.*[9], was also close to 90% complete. Final yield for both drugs based on weights, was slightly lower than expected from chromatography results, due mainly to losses during extraction.

Final labelled products were found to be reasonably pure. Major impurities such as residual reactants were probably minimized since reactions were monitored and allowed to proceed to completion. Nevertheless, prior to use in an immunoassay the labelled drug must be further purified, otherwise, traces of unconjugated drug or luminescent material could cause interference[8].

Some limitations have been observed with HPLC. It cannot be used for absolute quantitation of the reaction products unless standards are available to establish retention time and detector response. Preliminary synthesis followed by crystallization, is, therefore, still necessary to provide acceptably pure standards. Nevertheless, the speed and amount of information obtained by HPLC, with or without pure standards, exceed most other available systems.

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